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CONTRIBUTION OF EXTRA-ORAL TASTE RECEPTORS AND GUT HORMONES IN THE EFFECT OF SWEETENERS AND GASTRIC BYPASS SURGERY ON OBESITY

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I planned each charted course, each careful step along the byway
And more, much more than this, I did it my way
- Frank Sinatra

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ABBREVIATIONS

$^{-/-}$	Knockout
5-HT	5-hydroxytryptamine/serotonin
ADI	Acceptable daily intake
AGB	Adjustable gastric banding
AgRP	Agouti-related peptide
ALF	Ad libitum fed
ARC	Arcuate nucleus
AUC	Area under the curve
BMI	Body mass index
BPL	Biliopancreatic limb
CCK	Cholecystokinin
CCK1R	Cholecystokinin 1 receptor
CL	Common limb
CNS	Central nervous system
DNP	2,4-dinitrophenol
DNRI	Dopamine-norepinephrine reuptake inhibitor
DPP4	Dipeptidyl peptidase 4
EE	Energy expenditure
EEC	Enteroendocrine cell
ER	Extended release
FDA	Food and Drug Administration
FFAR	Free fatty acid receptor
FGF19	Fibroblast growth factor 19
FOS	Fructooligosaccharides
FXR	Ligand-activated transcription factor farnesoid-X-receptor
GABA	Gamma-aminobutyric acid
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1/2	Glucagon-like peptide 1/2
GLP-1R	Glucagon-like peptide 1 receptor
GLUT2	Glucose transporter 2
GOAT	Ghrelin-O-acyltransferase
GPCR	G protein-coupled receptor
HFD	High-fat diet

HIS	High-intensity sweetener
HOMA-IR	Insulin resistance index
IR	Immunoreactive
IP_3	1,4,5-inositol trisphosphate
K_{ATP} channel	ATP-sensitive K^+ channel
LPAR5	Lysophosphatidic acid receptor 5
MANOVA	Multivariate analysis of variance
MC4R	Melanocortin 4 receptor
MCT	Monocarboxylate transporter
MEMRI	Manganese-enhanced magnetic resonance imaging
MGN3-1	Mouse ghrelinoma cell line
NEFA	Non-esterified fatty acid
Ngn3	Neurogenin 3
NPY	Neuropeptide Y
OFS	Oligofructose
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PF	Pair-fed
$PLC\beta_2$	Phospholipase Cβ2
POMC	Pro-opiomelanocortin
PPARɣ	Peroxisome proliferator-activated receptor gamma
PVN	Paraventricular nucleus
PYY	Peptide YY
RGMS	Relative global market share
RIA	Radioimmunoassay
RL	Roux limb
RYGB	Roux-en-Y gastric bypass
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SGLT1	Sodium coupled glucose transporter
SMCT-1	Sodium-dependent monocarboxylate transporter 1
SNRI	Serotonin-noradrenalin reuptake inhibitor
SR	Sustained release
SREBP1	Sterol-regulatory element binding protein-1
T2DM	Type 2 diabetes
T_3	Tri-iodothyronine
T_4	Thyroxine

TAS1R	Taste receptor family type 1
TAS1R2-TAS1R3	Sweet taste receptor
TAS2R	Taste receptor family type 2
TASR	Taste receptor
TCA	Citric acid circle
TEER	Trans-epithelial electrical resistance
TGR5	Cell surface G-protein coupled receptor
TRPM5	Transient receptor potential cation channel subfamily M member 5
VSG	Vertical sleeve gastrectomy
WAT	White adipose tissue
WHO	World Health organization
WT	Wild type
Y2R	Y2 receptor
α-gust	α-gustducin
α-MSH	α-melanocortin-stimulating hormone

Chapter 1

INTRODUCTION

1 INTRODUCTION

1.1 Obesity

1.1.1 Introduction

Over the past four decades, we have transitioned from a world in which underweight prevalence was more than double that of obesity to one in which people are more obese than underweight (NCD et al., 2016). The World Health organization (WHO) estimates that, more than 1.9 billion adults were overweight in 2014, of which 600 million were obese (13% of the global population) (WHO, 2016).

Obesity is a chronic metabolic disease which results from an imbalance between energy uptake and energy expenditure (EE). In adults, obesity is defined by the body mass index (BMI). The BMI, originally named Quetelet index after its Belgian inventor, is calculated by dividing the body mass in kilograms by the square of the body height in meters. The BMI divides the population into underweight (BMI < 18.5), normal-weight (18.5 ≤ BMI < 25), overweight (25 ≤ BMI < 30), obese (30 ≤ BMI < 40) and morbidly obese (BMI ≥ 40) populations.

Obesity leads to an excessive accumulation of intra-abdominal fat, which can have adverse health consequences like the development of insulin resistance and type 2 diabetes (T2DM). The mechanisms linking obesity to insulin resistance involve several factors that are released by the adipose tissue like non-esterified fatty acids (NEFAs), glycerol, hormones, pro-inflammatory cytokines and other factors (Kahn et al., 2006). In obesity, the production of many of these products is increased, leading to insulin resistance. This insulin resistance can result in a decreased pancreatic insulin production which can impair adipocyte metabolism, resulting in increased lipolysis and elevated NEFA levels, which further fuel the development of T2DM. Thus the process may slowly feed forward, explaining why the onset of T2DM is usually a slow process that takes many years.
Obesity is not only linked to insulin resistance but also contributes to 33% of heart diseases, 50% of hypertensive incidents and 75% of diabetes prevalence (Forouzanfar et al., 2015). Associated with this, the life expectancy of morbidly obese patients is reduced by 10 years (Fontaine et al., 2003).

The comorbidities of obesity, such as cardiovascular disease ($193-315 billion) (Maahs et al., 2014) and T2DM ($105-245 billion) (Dall et al., 2009) result in medical obesity-related costs as high as $210 billion. This accounts for more than 20% of all annual healthcare spending in the US (Cawley and Meyerhoefer, 2012).

1.1.2 Origin

Obesity has reached epidemic proportions in populations whose environment promotes physical inactivity and increases consumption of energy dense, low-fiber foods. However, not all people in similar environmental conditions will become obese or suffer from the same health problems. Besides behavioral factors, genetics also determine our predisposition to become obese in an

obesogenic environment. Genetic studies in twins and families shows that genetic influences contribute up to 60% to abdominal adiposity (Wardle et al., 2008).
In rare cases (5%) obesity will not have a multifactorial, but a monogenetic cause. The absence of the leptin gene or leptin receptor for instance will cause obesity (Zhang et al., 1994). Other monogenetic obesity disorders are related to mutations in pro-opiomelanocortin (POMC) (Krude et al., 1998), the melanocortin 4 receptor (MC4R) (Vaisse et al., 1998) or prohormone convertase 1 (Jackson et al., 1997).

1.1.3 Treatment

Obesity treatment must begin with long-term lifestyle changes, including increased physical activity, dietary modifications using low-energy diets and behavioral therapy. Artificial sweeteners or functional food ingredients could be used as sugar replacers. When lifestyle changes alone are insufficient, pharmacotherapy can be added, although their side-effect profile and the modest nature of weight loss that can be achieved with these drugs should be taken into account. A last strategy for effective weight reduction is bariatric surgery.

1.1.3.1 Lifestyle changes

1.1.3.1.1 Lifestyle modification programs

A program for lifestyle modification includes diet, exercise and behavioral therapy and is considered the first step to achieve successful weight loss. How do we define 'successful weight loss management'? Wing and Hill proposed the following definition; '*An intentional weight loss of at least 10% of the body weight that keeps off for at least 1 year*' (Wing and Hill, 2001).

Dietary interventions; low-energy diets

A reduction of 500 kcal/day below energy requirements will provide health benefits (Ryan and Heaner, 2014). Many studies have focused on the amounts of dietary fat, protein and carbohydrates in the low-energy diets in an attempt to find the 'magic' weight-loss diet. However, these low-fat, low-carbohydrate or high-protein diets did not differ in their capacity to induce weight loss, indicating that the amount of calories is more important than the macronutrient composition of the diet (Johnston et al., 2014, Sacks et al., 2009). Therefore, the best practice is to provide low-energy diets that can be maintained by the patient and subsequently will provide health benefits.

Dietary interventions; artificial sweeteners

Sugar replacers, such as high-intensity sweeteners (HIS), can help to reduce the caloric content of meals without affecting its taste. So far sucralose (acceptable daily intake (ADI) 5 mg/kg/day, relative global market share (RGMS) 27.9%), aspartame (ADI 50 mg/kg/day, RGMS 27.9%), saccharin (ADI 15 mg/kg/day, RGMS 13.1%), acesulfame potassium (ADI 15 mg/kg/day, RGMS 5.2%), neotame (ADI 0.3

mg/kg/day, RGMS 1.4%) and advantame (ADI 32.8 mg/kg/day, RGMS 1.4%) have been approved by the Food and Drug Administration (FDA) as HIS (FDA, 2014, Research, 2011). Stevia has not yet been approved by the FDA as a HIS but has a RGMS of 8.7%. The global market for HIS totaled up to $13.5 billion in 2014. These products are mainly used in food products like soft drinks, coffee, breakfast cereals and chewing gum, but also in pharmaceutical products.

HIS are several times sweeter than sucrose (normal table sugar) but are non-caloric. Two opposite views exist on the link between HIS and obesity. The first view hypothesized that HIS might dysregulate appetite control due to the mismatch between the sweet taste and lack of consumed energy. This question was popularized by studies linking sweetened beverages and increased hunger ratings (Rogers and Blundell, 1989, Blundell and Hill, 1986, Rogers et al., 1988) and was underbuilt by epidemiological studies (Blum et al., 2005, Forshee and Storey, 2003). However, these epidemiological studies did not take dietary patterns and lifestyle modifications into account, leading to confounding results. Furthermore, no big randomized controlled trials were performed to support this hypothesis.
The second view stated that HIS are metabolically inert and might be used to decrease the caloric content of meals in order to decrease body weight. This hypothesis was underbuilt by studies that failed to link sweetened beverages and increased hunger ratings (Anderson et al., 1989, Rolls et al., 1990) and two big randomized controlled trials (de Ruyter et al., 2012, Tate et al., 2012) suggesting that replacing sugar-sweetened beverages with non-caloric beverages might be a useful strategy in the battle against obesity.

Dietary interventions; functional food ingredients

The current typical Western diet is generally lacking sufficient dietary fiber, being composed principally of refined grains and other highly digestible sources of starch, sugar, various fats, and animal products. Many adults in our Western society consume 5g to 10g of fiber daily, as opposed to the 35g to 50g that is recommended for optimal health (Lyon and Kacinik, 2012). Several studies also investigated the link between fiber intake and weight gain. A study in 252 women showed that for each 1g increase in total fiber consumed, weight decreased by 0.25kg (Tucker and Thomas, 2009). Four other cross-sectional reports reported inverse associations between cereal fiber intake and body weight measurements (Newby et al., 2007, Lairon et al., 2005, McKeown et al., 2009, van de Vijver et al., 2009). Furthermore, two prospective cohort studies found a dose-response relationship between fiber intake and weight gain (Koh-Banerjee et al., 2004, Du et al., 2010).

A specific kind of fibers, namely prebiotic sweeteners were proposed as functional food ingredients that may be useful to combat obesity. Prebiotics can be defined as; *'nondigestible compounds that, through their metabolization by microorganisms in the gut, modulate the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host*.' (Bindels et al.,

2015). The established prebiotics are; fructooligosaccharides (FOS) (oligofructose (OFS) and inulin), arabinoxylanoligosacharides, xylooligosacharides and galactooligosaccharides. OFS and inulin are derived from chicory root and differ in their polymerization degree; the average polymerization degree of OFS and inulin is estimated at 4 or 11 respectively although the polymerization degree of inulin can vary depending on its source and form.

These prebiotics are not absorbed in the small intestine of healthy individuals but are fermented by the gut microbiota to the short-chain fatty acids (SCFAs); acetate, propionate and butyrate (Gibson and Roberfroid, 1995). OFS and inulin can be used as natural sweeteners since they have a low caloric content (1.5 kcal/g) and a sweetening power of 30-35% in comparison to sucrose (table sugar) (Apolinario et al., 2014).

Supplementing OFS (10%) to a high-fat diet (HFD) for several weeks decreases body weight in rodents (Woting et al., 2015, Anastasovska et al., 2012, Delmee et al., 2006, Delzenne et al., 2005, Cani et al., 2005a). In contrast, a decrease (Cani et al., 2004a) or no effect (Parnell and Reimer, 2012) on body weight is observed when added to a normal chow diet. Moreover, OFS supplementation exerts an antidiabetic effect in streptozotocin-treated rats and HFD fed mice (Delzenne et al., 2007, Cani et al., 2006).

In humans the effect of the prebiotics on body weight is dose-dependent. In lean adults, 9g/day of inulin for 4 weeks did not affect body weight in a cross-over design (Brighenti et al., 1999). When they increase their daily OFS intake gradually every week during 5 weeks from 15g to 55g, >35g OFS/day suppressed hunger without altering energy intake (Pedersen et al., 2013). In prediabetic patients, 30g/day inulin promoted weight loss during an 18 week study period (Guess et al., 2015), while in overweight adults contradictory results have been reported. Some studies investigating the effects of OFS or inulin reported a reduced body weight (Yang et al., 2012, Hoeger et al., 1998, Parnell and Reimer, 2009), while body weight or energy intake was not altered in others (Savastano et al., 2014, Daud et al., 2014). In overweight and obese children, 12 weeks OFS supplementation did not affect body weight in a randomized, double-blind, placebo controlled trial (Liber and Szajewska, 2014).

The positive effects of these prebiotics on energy homeostasis have been assigned to their fermentation products, namely the SCFAs. Indeed, SCFAs may directly affect adiposity by influencing lipogenesis and stimulating fatty acid oxidation (den Besten et al., 2015, Gao et al., 2009, Lu et al., 2016). Furthermore, SCFAs can regulate the release of gut hormones involved in appetite regulation (Lin et al., 2012, Freeland and Wolever, 2010). Acetate may also play a role in central appetite regulation since; 1) ^{13}C acetate preferentially accumulates in the hypothalamus, 2) a direct intracerebroventricular administration of acetate into the third ventricle suppressed food intake in rats, 3) an intraperitoneal injection of acetate increased signal intensity, measured via manganese-enhanced magnetic resonance imaging (MEMRI), in the arcuate nucleus (ARC) and 4) an intraperitoneal injection increased POMC and reduced agouti-related peptide (AgRP) expression

(Frost et al., 2014). SCFAs have also been shown to improve glucose homeostasis and insulin sensitivity via activation of intestinal gluconeogenesis (De Vadder et al., 2014), improvement of insulin resistance (Gao et al., 2009) and stimulation of glucagon-like peptide 1 (GLP-1) secretion (Cani et al., 2006).

Dietary intervention studies in humans indicate that SCFAs could regulate energy intake and body weight. This has been studied in a randomized-controlled 24-week trial in 60 overweight adults, where an inulin-propionate ester was used to specifically deliver propionate to the colon at concentrations that cannot be reached by unpalatably high levels of fermentable dietary fibers alone. Daily supplementation of 10g of this ester significantly reduced weight gain, intra-abdominal adipose tissue distribution, intrahepatocellular lipid content and prevented the deterioration in insulin sensitivity compared to the inulin-control group (Chambers et al., 2015).

Physical activity

Increasing physical activity is another essential component of lifestyle modification programs. Recommendations in the US and UK recommend 150min/week of physical activity (National et al., 2014). Although more physical activity (30-45min/day) is needed to prevent obesity (Ryan and Heaner, 2014) and 60-90min/day may be needed to avoid weight regain (Ryan and Heaner, 2014). Furthermore, using physical activity as a weight-loss strategy resulted in a lower relapse and body weight regain in a follow-up study (Kayman et al., 1990, Leser et al., 2002). Therefore physical activity appears to play an important role in the maintenance of long-term weight loss.

Behavioral therapy

Behavioral therapy refers to a combination of techniques that help obese patients to modify their eating pattern, activity and thinking habits to control their excessive weight. Some of the core strategies of behavioral therapy are; self-monitoring, stimulus control, problem solving, cognitive restructuring and relapse prevention training (Diabetes et al., 2002).

The benefit of lifestyle modification programs

A systematic review on the outcome of weight loss induced by lifestyle modification programs showed that, in case of intense interventions, at 1 year only 28% of the participants obtained a weight loss ≥ 10%, 26% decreased their body weight with 5-9.9% and 38% with ≤ 4.9%. In less intense interventions 13% lost ≥ 10%, 16% decreased their body weight with 5-9.9% and 27% lost ≤ 4.9% of their body weight (Christian et al., 2010). '*Intense lifestyle modification interventions' were defined as those in which participants were asked to attend sessions where the intervention was delivered for an average of 37 h, whereas 'less intense lifestyle modification interventions' were those where sessions required participants to attend an average of 5 h* (Christian et al., 2010). Furthermore, although the weight loss after these programs reached its peak within 6 months after the start of the treatment,

the trend began to reverse thereafter, with 30% and 50% returning to their original weight after 1 and 5 years respectively (Curioni and Lourenco, 2005).
Despite this modest weight loss, lifestyle modification programs can prevent the onset of T2DM with 58% (Knowler et al., 2002). Moreover, during a follow-up study of 15 years, diabetes incidence was still reduced by 27% in the lifestyle intervention group, despite the weight regain (Prevention et al., 2015).

1.1.3.2 Pharmacological treatment

Currently lifestyle modifications are regarded as the cornerstone for the prevention and management of obesity. However, the addition of weight-reducing therapies could help to induce and maintain body weight loss.
The indications for adding pharmacotherapy to a weight loss effort are; a BMI ≤ 27 with one or more comorbidities or a BMI ≥ 27 (Apovian et al., 2015). A pharmacological treatment is deemed effective when a weight loss of 5% or more is achieved within 3 months after the start of the treatment. When this criterion is not met, it is recommended that the pharmacotherapy is discontinued and alternative drugs or referral for alternative treatment approaches be considered.

Orlistat is currently the only anti-obesity drug that can be used on a long-term basis. Orlistat is a potent and selective inhibitor of gastric and pancreatic lipases (Sternby et al., 2002). Thus it reduces the subsequent absorption of monolglycerides and free fatty acids. Orlistat is approved for over-the counter use, despite its notorious gastrointestinal side effects such as steatorrhea. Diet pills containing 2,4-dinitrophenol (DNP) increased metabolic rate but were withdrawn from the market due to their severe side-effect profile which included agranulocytosis, dermatitis and fatal hyperthermia. Aminorex, an amphetamine-like drug was withdrawn since it caused chronic pulmonary hypertension. Fen-Phen, which is a combination of fenfluramine, a serotonin-noradrenalin reuptake inhibitor (SNRI) and phentermine, an amphetamine derivative, was withdrawn from the market due to its association with valvular heart disease. Rimonabant, a cannabinoid receptor 1 antagonist, was previously available but was withdrawn due to an increased risk in depression and suicidality. Sibutramine, a SNRI, induced cardiovascular risks (Bray et al., 2016).
From 2012 on a repertoire of new therapeutic agents to treat obesity became available, which included the 4 drugs (Table 1.1); lorcaserin, phentermine/topimarate, naltrexone/bupropion and liraglutide. Lorcaserin in a small-molecule agonist of the serotonin 2C (5-HT_{2C}) receptor in the central nervous system (CNS). Specifically it stimulates 5-HT_{2C} receptors on the POMC neurons in the ARC, which causes the release of α-melanocortin-stimulating hormone (α-MSH) to suppress appetite. Phentermine is a sympathomimetic amine which also acts centrally as an appetite suppressant. Topimarate is a sulfamate-substituted monosaccharide which was initially approved by the FDA for management of seizure disorders, its mechanisms of action for obesity remains unclear. Naltrexone is an opioid agonist while bupropion is a dopamine reuptake inhibitor. Liraglutide can induce body

weight loss by working as a long-acting GLP-1 receptor (GLP-1R) agonist (Bray et al., 2016). Interestingly these new agents are drugs that were previously used for other indications. Some of them also pose an extensive side-effect profile (Table 1.1). These drugs can induce at least 5% weight loss after 52 weeks (Table 1.1).

The only anti-obesity drugs on the European market are currently orlistat, naltrexone/bupropion and liraglutide (Table 1.1). The central working anti-obesity drugs lorcaserin and phentermine/topimarate are not yet approved by the European Medicine Agency.

	Mechanism of action	Available for chronic use		Mean percentage weight loss		Advantages	Disadvantages
		USA	European Union	Placebo	Drug		
Phentermine	Sympathomimetic	Short-term use	No	Not stated	Not stated	Inexpensive	Side-effects No long-term data
Orlistat	Gastric and pancreatic lipase inhibitor	Yes	Yes	-2.6%	-6.1%	Not absorbed	Side-effects Modest weight loss
Lorcaserin	5-HT2c seretonin agonist	Yes	No	-2.5%	-5.8%	Mild side-effects	Expensive Modest weight loss
Phentermine/ topimarate ER	Sympathomimetic anticonvulsant	Yes	No	-1.2%	-7.8%	Robust weight loss	Expensive Teratogen
Naltrexone SR/ bupropion SR	Opioid receptor antagonist/ DNRI	Yes	Yes	-1.3%	-5.4%	Reduces food cravings	Side-effects Expensive
Liraglutide	GLP-1 receptor agonist	Yes	Yes	-3%	-7.4%	Mild side-effects	Expensive Injectable

Table 1.1 Pharmacological treatment of obesity. Drugs for weight management with their mechanisms of action, availability, induced effect, advantages and disadvantages (Adapted from Bray, Lancet, 2016). Abbreviations: 5-HT, 5-hydroxytryptamine/serotonin; DNRI, dopamine-norepinephrine reuptake inhibitor; ER, extended release; SR, sustained release.

1.1.3.3 Bariatric surgery

1.1.3.3.1 General

Bariatric surgery is generally considered when nonsurgical interventions have failed in patients with a BMI of ≥35 kg/m^2 with one or more comorbidities, or a BMI of ≥40. Bariatric surgery remains the most effective and durable therapeutic option for obesity. Common bariatric surgeries include adjustable gastric banding (AGB), vertical sleeve gastrectomy (VSG), and Roux-en-Y gastric bypass (RYGB) surgery. In 2011, the global percentage of each of those bariatric procedures was as followed; AGB 17.8%, VSG 27.8% and RYGB 46.6% (Buchwald and Oien, 2013).

AGB involves placing a silicone ring around the top of the stomach. VSG is a bariatric procedure that reduces the stomach to about 15% of its original size, by surgically removing a portion of the stomach along the greater curvature. During RYGB surgery a small pouch is created under the esophagus and the remaining stomach and part of the small intestine are bypassed by connecting the jejunum directly to the esophagus. The remaining stomach and small intestine are reconnected with a lower part of the jejunum (Figure 1.1) (Piche et al., 2015).

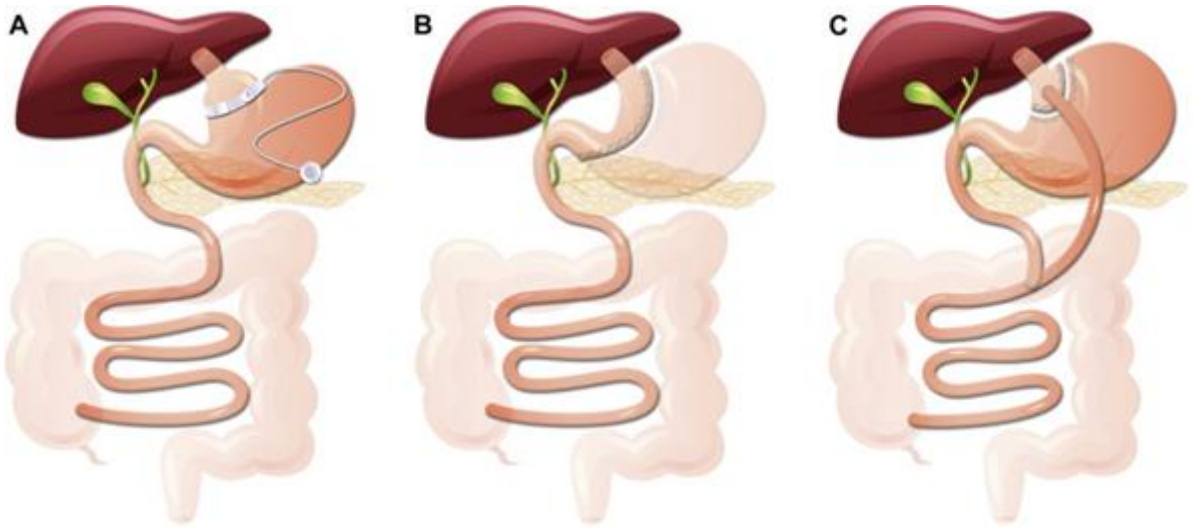


Figure 1.1 Postsurgical anatomy. The postsurgical anatomy after (A) AGB, (B) VSG and (C) RYGB (Adapted from Piché, Can J Cardiol., 2015). Abbreviations: AGB, Adjustable gastric banding; RYGB, Roux-en-Y gastric bypass surgery; VSG, Vertical sleeve gastrectomy.

The mean weight loss after these surgical techniques was investigated in the Swedish Obese Subjects study involving 4047 obese subjects of which 2010 underwent bariatric surgery and 2037 received conventional treatment (matched control group). In this study, weight loss was maximal after 1-2 years with on average 20% weight loss after AGB, 25% after VSG and 32% after RYGB. After this initial drop, patients started to regain weight but this weight regain leveled off after 8-10 years. These procedures resulted in a stable weight loss of 14% for AGB, 16% for VSG and 25% for RYGB (Sjostrom et al., 2007).

The beneficial effects of bariatric surgery (AGB, VSG and RYGB) compared to the other treatment strategies are not exclusively limited to weight loss but also include resolution of comorbidities (T2DM, dyslipidemia, hypertension, sleep apnea) associated with obesity (Patkar et al., 2016). For instance, bariatric surgery reduces the long-term incidence of T2DM by 78% in obese patients (Carlsson et al., 2012). A randomized non-blinded study showed that 37% of the patients in the VSG group and 42% of the patients in the RYGB group reduced their glycated hemoglobin levels to at least 6%. In contrast, only 12% of the patients in the medical therapy group (which received lifestyle counseling, weight management, frequent home glucose monitoring and the use of newer approved drug therapies) succeeded in lowering their glycated hemoglobin levels to at least 6% (Schauer et al., 2012).

1.1.3.3.2 Mechanisms of action

The mechanisms behind the metabolic improvement after bariatric surgery are not fully elucidated yet. Some of the proposed mechanisms are; an altered energy expenditure (EE), food restriction, food malabsorption, altered gut hormone release, altered bile acid secretion and central effects. These effects are summarized in Figure 1.2.

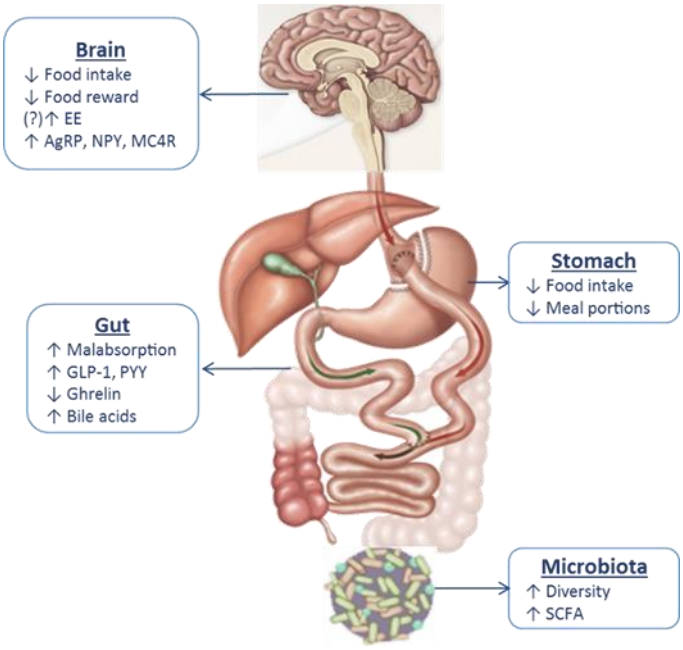


Figure 1.2 Potential mechanisms of the metabolic improvements after RYGB surgery. Role of EE, food restriction, food malabsorption, gut hormones, bile acids and central melanocortin signaling in the metabolic improvements after RYGB surgery. Abbreviations: AgRP, agouti-related peptide; EE, energy expenditure; GLP-1, glucagon-like peptide 1; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; PYY, peptide YY; SCFA, short-chain fatty acid.

1.1.3.3.3 Energy expenditure

The EE is the sum of the basal metabolic rate, the thermic effect of feeding and the EE of physical activity. The difference between the rates of EE and energy intake defines energy balance.

Human studies conclude that bariatric surgery decreases EE (Das et al., 2003, Carrasco et al., 2007, Coupaye et al., 2005). But still, these results remain controversial since it is quite difficult to compare different weights and body compositions with their relative rates of EE, as weight loss itself will decrease EE. Therefore the question should be posed as; will the effect on EE seen after RYGB surgery be different compared to a similar amount of weight loss imposed in another manner?

1.1.3.3.4 Restriction

Since AGB, VSG and RYGB are all mechanically restrictive techniques, it was originally hypothesized that making a small pouch would physically limit the amount of calories that could be consumed over short intervals. Thus an increased gastric pressure would trigger early satiety. However, recent studies clearly showed that although AGB, RYGB ad RYGB are all restrictive techniques, the reduction in stomach size is not the main cause for weight loss after these procedures. This has been supported by several lines of evidence. First of all, Topart *et al.* showed that the pouch size after gastric bypass does not correlate with weight loss outcomes (Topart et al., 2011). Secondly, the stomach volume after VSG in humans (150-200ml) is larger than the volume remaining after gastric banding (12-20ml) (Gagner and Rogula, 2003), although VSG induces a greater weight loss than gastric banding (Sjoholm

et al., 2013). Thirdly, gastric dilatation after VSG does not abrogate the weight loss after this procedure (Langer et al., 2006). Finally, after VSG (Stefater et al., 2010) and RYGB surgery (Zheng et al., 2009), much smaller and more frequent meals are consumed, therefore meal size will not limit the total daily caloric intake.

1.1.3.3.5 Malabsorption

RYGB does not only restrict stomach size but also bypasses a part of the absorptive capacity of the intestine, which may result in food malabsorbtion. This malabsorption may contribute to the profound weight loss after RYGB, but it also puts patients at a higher risk for micronutrient deficiencies (Bal et al., 2012, Hammer, 2012). Other classic malabsorptive techniques are the biliopancreatic diversion and the biliopancreatic diversion with duodenal switch. These procedures have by far the highest long-term complication rates and result in caloric, protein and micronutrient deficiencies (Aasheim et al., 2009, Billeter et al., 2014). Most bariatric procedures try to minimize these effects, with clinical studies reporting no, or minimal nutrient malabsorption (sugar and fat) after bariatric procedures such as RYGB surgery (Wang et al., 2012, Carswell et al., 2014, Kumar et al., 2011).

VSG, which is a restrictive, but not a malabsorptive technique, induces comparable weight loss compared to RYGB surgery (Sjostrom et al., 2007). This suggests that macronutrient malabsorption plays a minor role in the weight loss induced by RYGB or that the mechanisms of weight loss after VSG and RYGB differ.

1.1.3.3.6 Gut hormones

Altered release of gut hormones is considered as one of the leading candidate mechanisms for the metabolic improvement after RYGB surgery. More specifically, RYGB surgery enhances the secretion of the anorexigenic hormones GLP-1 and peptide YY (PYY) and, although more controversial, inhibits secretion of the orexigenic hormone ghrelin. Figure 1.3. depictures the central mechanisms through which gut hormones may induce orexigenic and anorexigenic effects.

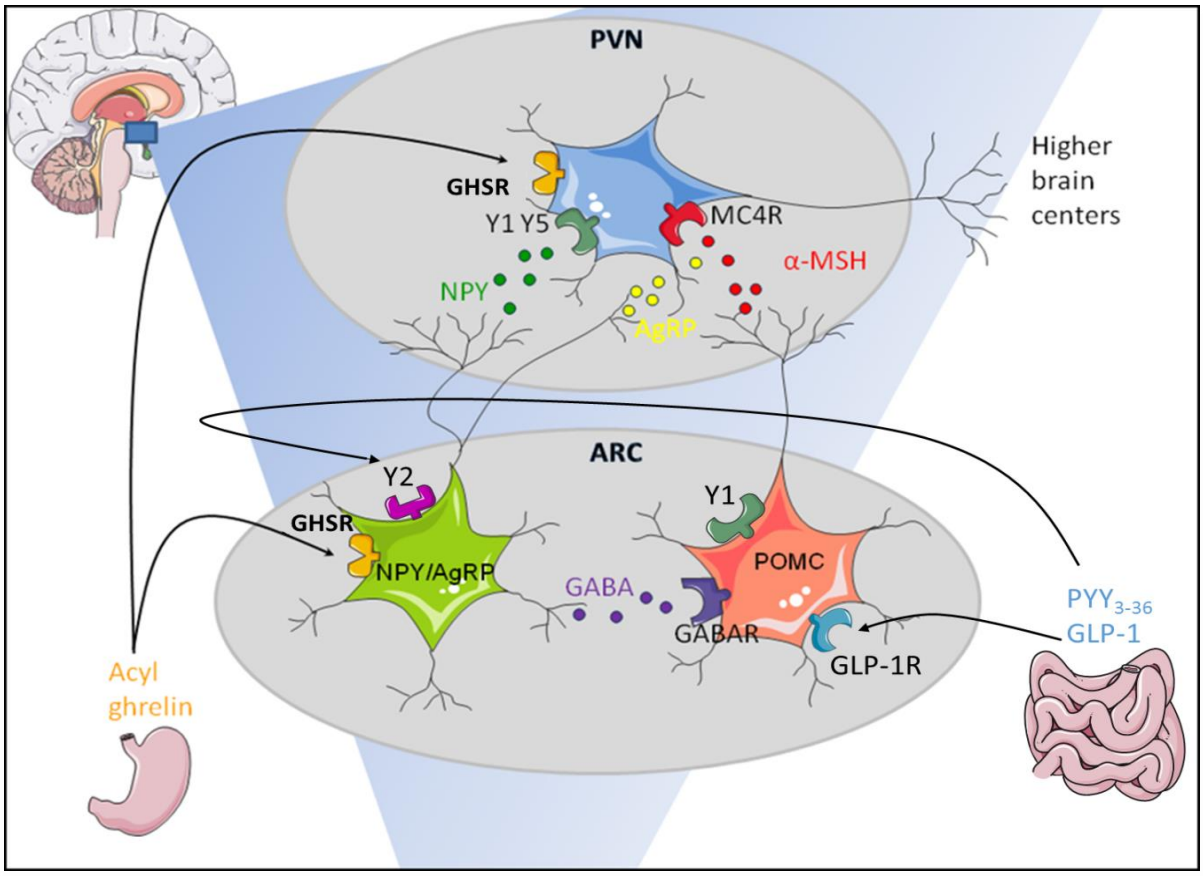


Figure 1.3 Mechanisms underlying the orexigenic effect of ghrelin and the anorexigenic effect of GLP-1 and PYY. Ghrelin stimulates food intake and GLP-1 and PYY inhibit food intake by acting on hypothalamic circuits in the ARC and paraventricular nucleus (PVN) (Adapted from Briggs and Andrews, Neuroendocrinology, 2010). Abbreviations: ARC, arcuate nucleus; GABA, gamma-aminobutyric acid; GHSR, growth hormone secretagogue receptor; GLP-1R, glucagon-like peptide 1 receptor; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus.

Ghrelin

Ghrelin is mostly (80%) secreted by X/A cells from the stomach. Ghrelin is a 28-amino acid peptide with a unique posttranslational modification consisting of an octanoyl group at Ser^3 which is necessary for the activation of its receptor (growth hormone secretagogue receptor; GHSR) and thus for its biological activity. The octanoylation is catalyzed by ghrelin O-acyltransferase (GOAT), a member of the 16 hydrophobic membrane bound acyltransferase family (Muller et al., 2015).

Physiological functions of ghrelin

Besides its effect on growth hormone release, the most important function of ghrelin is the stimulation of food intake and its role in energy metabolism. Plasma ghrelin levels rise preprandially and decrease after the meal, indicating that ghrelin is a meal-initiating factor. Indeed, both peripheral and central administration of ghrelin dose-dependently increased food intake in rodents.

In humans an intravenous or subcutaneous administration of ghrelin also increased food intake and enhanced hunger scores (Muller et al., 2015).

Ghrelin is believed to exert its biological functions on food intake through its receptor present in the ARC of the hypothalamus. A humoral and neural pathway has been suggested for the central activation of the ghrelin receptor. The neural pathway suggests that ghrelin can stimulate food intake indirectly via its receptor on the vagal nerve. GHSRs are synthesized in the neuronal cell bodies in the nodose ganglion and transported to the nerve terminals through axonal transport (Date et al., 2002). In rats 75% of GHSR containing neurons in the nodose ganglion express other orexigenic receptors (cannabinoid and melanin-concentrating hormone receptors) and anorexigenic receptors (cholecystokinin; CCK and leptin receptor) (Burdyga et al., 2006, Date et al., 2005, Burdyga et al., 2002). Furthermore, the CCK-mediated upregulation of the cocaine- and amphetamine-regulated transcript (which inhibits food intake) in vagal afferent neurons, was inhibited by ghrelin (de Lartigue et al., 2007). Ghrelin also selectively inhibited subpopulations of mechanically sensitive gastroesophageal vagal afferents but the subpopulations of afferents inhibited differed between mice and ferrets (Page et al., 2007).

The role of the vagus nerve in the orexigenic effects of ghrelin is controversial but it is generally accepted that this orexigenic effect is at least partially mediated through GHSR activation on the vagus nerve. Truncal vagotomy and vagal afferent ablation or perivagal application of capsaicin, but not subdiaphragmatic vagal differentiation in rodents abolished the stimulatory effect of peripherally administered ghrelin on food intake (Asakawa et al., 2001, Date et al., 2002, Arnold et al., 2006). Furthermore, ghrelin was unable to stimulate food intake in humans who underwent surgery involving vagotomy (le Roux et al., 2005). The expression of GHSR on the vagal nerve may play an important physiological role in the setting of excessive nutrition, since its expression in vagal afferents is increased in diet-induced obese rats (Paulino et al., 2009). This may maintain ghrelin signaling during obesity, when ghrelin levels are decreased (Kentish et al., 2013).

The humoral pathway suggests that ghrelin released from the stomach can directly, via the bloodstream, activate the ghrelin receptor on the orexigenic neuropeptide Y (NPY)/AgRP neuron in the ARC. The activation of this neuron by ghrelin has been demonstrated by electrophysiological recording studies on hypothalamic slices (Cowley et al., 2003). Furthermore, chronic central administration of ghrelin resulted in a higher expression of NPY and AgRP, which stimulated food intake (Kamegai et al., 2001). The effect of ghrelin on food intake was completely abolished in double knockout mice for NPY and AgRP (Chen et al., 2004). This indicated that NPY and AgRP are both necessary for the orexigenic effects of ghrelin. NPY can activate Y1/Y5 receptors in the PVN to stimulate food intake. AgRP is an antagonist of the MC4R containing neurons in the PVN which inhibit food intake. Additionally, ghrelin also indirectly inhibits the activity of POMC neurons by stimulating gamma-aminobutyric acid (GABA) release from NPY/AgRP neurons (Cowley et al., 2003).

The ghrelin receptor is also expressed in tegmental and mesolimbic systems that are involved in reward (Zigman et al., 2006). Brain imaging studies in humans showed that intravenous administration of ghrelin stimulated brain regions involved in reward processing and appetitive behavior (Malik et al., 2008).

Besides initiating food intake and influencing hunger on a short-term basis, ghrelin also promotes fat storage resulting in an increased body weight on the long term (Nakazato et al., 2001, Tschop et al., 2000, Wortley et al., 2004). The effect of ghrelin on adiposity is partially regulated via ghrelin receptors in the CNS (Heppner et al., 2014) where ghrelin can regulate the sympathetic nervous system activity to influence adiposity (Theander-Carrillo et al., 2006).

Previous data also indicate that ghrelin has direct peripheral effects on adipocyte and lipid metabolism; 1) intravenous ghrelin administration in rats increased white adipose tissue (WAT) mass by inducing lipid retention in adipocytes (Davies et al., 2009), 2) ghrelin reduced TNF-α-induced apoptosis and autophagy in human visceral adipocytes *in vitro* (Rodriguez et al., 2012) and 3) ghrelin stimulated lipid accumulation in cultured human omental adipocytes *in vitro* by targetting several fat storage-related proteins (Rodriguez et al., 2009, Choi et al., 2003).

Surprisingly, obese people show significantly lower ghrelin levels compared to lean people (Tschop et al., 2001). This probably represents a positive feedback signal to the positive energy balance. The only exception to this is the Prader-Willi syndrome, a genetic obesity syndrome which is characterized by severe obesity and hyperphagia. These patients have 3-fold increased ghrelin levels (Goldstone et al., 2004). Obese patients do not only have a lower basal ghrelin release but also show a less pronounced drop in ghrelin levels after food intake which can lead to a persistent hunger signal and increased food intake (English et al., 2002). In contrast, ghrelin levels are elevated in anorexia patients. These ghrelin levels may reflect its compensating role in energy balance.

Ghrelin and bariatric surgery

The role of ghrelin in energy metabolism makes altering postprandial ghrelin levels a plausible candidate mechanism for the metabolic improvements after bariatric surgery. However, controversial findings have been reported concerning the effect of bariatric surgery on ghrelin levels. The first study reported a dramatic decrease in plasma ghrelin levels after RYGB surgery in humans (Cummings et al., 2002). This was confirmed in some studies but not in others which reported no change or an increase in ghrelin levels (Meek et al., 2016). Furthermore, studies using ghrelin deficient mice have shown that the effects of VSG on the metabolic improvements are ghrelin independent (Chambers et al., 2013). These results indicate that alterations in ghrelin levels may play a minor role in the metabolic improvements after bariatric surgery.

GLP-1

GLP-1 is secreted from intestinal L-cells and the biological active hormones GLP-$1_{7\text{-}37}$ and GLP-$1_{7\text{-}36\ amide}$ are rapidly degraded by the enzyme dipeptidyl peptidase 4 (DPP4) to antagonists (Kieffer et al., 1995). GLP-$1_{7\text{-}37}$ and GLP-$1_{7\text{-}36\ amide}$ are equally potent at activating the GLP-1 receptor.

Physiological functions of GLP-1

GLP-1 has numerous physiological actions, including potentiation of glucose-stimulated insulin secretion through its binding with the GLP-1 receptor on pancreatic β-cells, β-cell proliferation and inhibition of apoptosis, inhibition of glucagon release, delaying gastric emptying and inhibiting food intake (Steinert et al., 2016).

The biological functions of GLP-1 made it an ideal therapeutic target to treat T2DM. Since native GLP-1 has a short half-life, novel methods have been developed to exploit its anti-hyperglycemic effects. They can broadly be classified in short-and long working GLP-1 analogues and DPP4 inhibitors. Currently, the GLP-1 analogue liraglutide is approved for the treatment of obesity and several other drugs are in the pipeline for this indication (Isaacs et al., 2016).

The GLP-1R is not only present in the pancreas, but also in the hypothalamic ARC and PVN (Wei and Mojsov, 1995). GLP-$1_{7\text{-}36\ amide}$ induced c-fos activation in the PVN of rodents (Larsen et al., 1997b, Baggio et al., 2004a) and changed neuronal activity in the prefrontal cortex and hypothalamus of humans (Pannacciulli et al., 2007). There have been contradictory findings on whether or not GLP-1 can cross the blood brain barrier to directly act on these brain regions. Although it has been shown that GLP-1 can gain access to these brain regions through simple diffusion (Kastin et al., 2002), a fusion protein of albumin and GLP-1 that cannot pass the blood brain barrier can also reduce feeding (Baggio et al., 2004b), suggesting that GLP-1 reduces feeding via GLP-1Rs in nodose ganglia. In addition, the fact that circulating GLP-1 is rapidly degraded by DPP4 argues against activation of the GLP-1R in the CNS by peripheral GLP-1 (Mentlein et al., 1993). Furthermore, central GLP-1R blockade did not affect GLP-1-induced anorexia (after intraperitoneal administration) suggesting that central GLP-1R activation did not play a role in this effect (Williams et al., 2009).
The role of the vagus nerve in the satiating effects of GLP-1 has been supported by a study in rodents that reported no effect of peripheral administration of GLP-1 on food intake and activation of hypothalamic ARC neurons after either bilateral subdiaphragmatic total truncal vagotomy or brainstem-hypothalamic transsectioning in rodents (Abbott et al., 2005). Furthermore, capsaicin pretreatment prevented the inhibitory effect of Exendin-4 (a GLP-1R agonist) on food intake (Talsania et al., 2005).

The GLP-1R does not only play a role in the anorexigenic effect of GLP-1, but might also play a role in anticipatory food reward. This has been suggested by a study where Exendin-4 increased brain responses to receipt of chocolate milk and decreased anticipation of chocolate milk compared with placebo. Furthermore, a GLP-1R antagonist largely prevented these brain responses to chocolate milk compared with placebo, suggesting that GLP-1R activation plays a role in anticipatory food reward (van Bloemendaal et al., 2015).

GLP-1 and bariatric surgery

Postprandial GLP-1 levels are dramatically increased after both VSG and RYGB in rodents and humans and this postprandial increase persists over several years (Meek et al., 2016). However, studies in GLP-1R$^{-/-}$ mice provided no support for a role of GLP-1 in the body weight-lowering effects of VSG and RYGB (Ye et al., 2014, Wilson-Perez et al., 2013, Mokadem et al., 2014). In contrast, the decreased appetite after RYGB in humans returned when the GLP-1 release was inhibited (le Roux et al., 2007, Svane et al., 2016), suggesting an important role for GLP-1 in the RYGB-induced weight loss in humans.

PYY

PYY is also secreted from intestinal L-cells. Full-length PYY acts on the Y-family receptors Y1, Y2, and Y5, whereas digestion of $PYY_{1–36}$ by DPP4 results in the generation of $PYY_{3–36}$, a selective agonist for the Y2 receptor (Y2R) (Rose et al., 1995).

Physiological functions of PYY

PYY can affect satiety by delaying gastric emptying (Savage et al., 1987) and by inducing the ileal brake (Lin et al., 1996). Circulating PYY_{3-36} affects the CNS directly after transport across the blood brain barrier (Gustafson et al., 1997), or by activation of the vagus-brainstem-hypothalamic pathway (Koda et al., 2005). In the CNS, studies in Y2R$^{-/-}$ mice showed that peripheral PYY stimulates satiety by activating Y2Rs on the NPY/AgRP neuron and by increasing activity of the anorectic POMC/α-MSH neuron (Batterham et al., 2002). However, this effect of peripheral PYY administration could not be repeated by other laboratories (Boggiano et al., 2005). In humans, the therapeutic window of PYY to inhibit food intake is small since adverse effects (like nausea) might mediate also mediate the inhibition of food intake. Therefore it is difficult to distinguish between the genuine satiating effect of PYY and the reductions in appetite due to nausea (Perry and Wang, 2012). Furthermore, studies using functional magnetic resonance imaging (MRI), showed that PYY_{3-36} can switch the regulation of food intake from a homeostatic brain region (hypothalamus) to a hedonic brain region (Batterham et al., 2007).

The Y2R is also synthesized in the rat nodose ganglion and transported to the vagal afferent terminals (Koda et al., 2005). Abdominal vagotomy abolished the anorectic effect of PYY_{3-36} and the PYY_{3-36} induced c-fos expression in the ARC of rats (Koda et al., 2005), suggesting a role for the vagal nerve in the anorectic effect of PYY_{3-36}.

PYY and bariatric surgery

Postprandial PYY levels are rapidly increased after bariatric surgery and persist after one year. Several types of bariatric surgery increase PYY levels, such as AGB, VSG and RYGB (Meek et al., 2016). Furthermore, the effects of bariatric surgery on body weight loss were absent in $PYY^{-/-}$ mice (Chandarana et al., 2011). Nevertheless, central infusion of the selective Y2R-antagonist BIIE0246 had no effect on the body weight loss induced by RYGB surgery in mice, suggesting that the Y2R does not mediate the effects of RYGB surgery on body weight (Ye et al., 2014). In contrast, a human study showed that combined pharmacological blockage of GLP-1 and PYY_{3-36} increased food intake after RYGB, supporting the hypothesis that these hormones have a role in the decreased postoperative food intake. This study also revealed that blocking the actions of only one of these hormones resulted in the increased secretion of the other, explaining previous findings questioning the role of GLP-1 and PYY in the decreased food intake and body weight loss after RYGB (Svane et al., 2016).

Other hormones have been shown to be altered after bariatric surgery, including CCK, glucose-dependent insulinotropic peptide (GIP), glucagon and GLP-2 (Meek et al., 2016). Determining the relative contribution of these different hormones in the postsurgical weight loss remains an important research goal.

1.1.3.3.7 Bile acids

Bile acids are potent digestive surfactants that promote lipid absorption. Primary bile acids are synthesized in the liver and duodenally secreted in response to fatty acid ingestion. In addition to their role in lipid absorption, bile acids can also act in a 'hormone-like' manner. They are the natural ligand for several receptors amongst which; a cell surface G-protein coupled receptor (TGR5) (Maruyama et al., 2002, Kawamata et al., 2003) and a ligand-activated transcription factor farnesoid-X-receptor (FXR) (Wang et al., 1999) via which they play an important role in the energy-and glucose homeostasis.

Postprandial bile acid levels correlate inversely with BMI (Suzuki et al., 2014). Furthermore, supplementing a HFD with cholic acid (a bile acid) increases EE, oxygen consumption, prevents obesity and decreases insulin resistance in a mice model of obesity (Watanabe et al., 2006). *In vitro* experiments in brown adipocytes and skeletal myocytes suggest that these effects are mediated through increased cAMP production after TGR5 activation. This in turn will activate the key enzyme iodothyronine deiodinase, which converts inactive thyroxine (T_4) into intracellular active tri-iodothyronine (T_3), a major component in cellular basal metabolism (Watanabe et al., 2006). The bile acid-induced protection against insulin resistance has been attributed to TGR5 mediated GLP-1 release (Thomas et al., 2009, Duboc et al., 2014). Furthermore, FXR activation can improve glycemic control and increase EE by increasing the concentration of fibroblast growth factor 19 (FGF19) (Kir et al., 2011, Tomlinson et al., 2002).

Serum bile acids are elevated after RYGB surgery and VSG (Cole et al., 2015, Steinert et al., 2013), normalizing the blunted postprandial bile acids concentrations in obesity (Kohli et al., 2010). The increased bile acid levels after bariatric surgery could contribute to the increased postsurgical plasma GLP-1 levels through TGR5 activation on L-cells (Thomas et al., 2009, Duboc et al., 2014). Furthermore, experiments in WT and FXR knockout mice showed that FXR plays an important role in the effect of VSG on body weight and glucose tolerance (Ryan et al., 2014), suggesting that altered bile acid signaling may play a role in the metabolic improvements after bariatric surgery.

1.1.3.3.8 Central control of energy balance

The brain responds to internal cues from the periphery and relays information about long-term and short-term energy availability to regulate body weight maintenance. The ARC of the hypothalamus is an important component of this homeostatic system. The ARC contains catabolic POMC-neurons and anabolic NPY and AgRP-neurons. POMC is cleaved to produce α-MSH, a hormone which decreases food intake and induces weight loss when administered exogenously (Tsujii and Bray, 1989). Furthermore, the AgRP is an antagonist of the MC4R which is found in several brains regions such as the PVN via which it can induce feeding and weight gain (Fan et al., 1997). Like AgRP, NPY stimulates food intake and weight gain (Morley et al., 1987).

Given the importance of central melanocortin signaling in energy homeostasis, the decrease in body weight after bariatric surgery may result from changes in this axis. RYGB is still effective in patients with a heterozygous mutation in the gene encoding MC4R (Aslan et al., 2011), suggesting that one functional copy of MC4R is sufficient to permit the effect of RYGB on energy intake. Importantly, carriers of a MC4R variant known to increase basal MC4R activity show greater weight loss after RYGB compared to noncarriers of this variant (Mirshahi et al., 2011). In contrast to VSG (Stefater et al., 2010), RYGB surgery in rats increased mRNA levels of the orexigenic neuropeptides AgRP and NPY in the ARC, whereas no change was observed in anorexigenic POMC mRNA levels. This suggests that

RYGB surgery puts a brake on orexigenic hypothalamic output signals (Barkholt et al., 2016). Furthermore, $MC4R^{-/-}$ mice showed that MC4R function is critical for the sustained reductions in food intake and weight loss induced by RYGB (Hatoum et al., 2012). Intracerebroventricular administration of a specific MC4R antagonist in sham and RYGB rats led to a doubling of food intake and weight regain (Mumphrey et al., 2014). MC4R may also be directly involved in the regulation of PYY and GLP-1 secretion, since the MC4R is expressed on enteroendocrine L-cells and intraperitoneal administration of melanocortin peptides in mice stimulate PYY and GLP-1 release (Panaro et al., 2014). However, until know it is unclear if central affects play a causal role in the weight loss after bariatric surgery, or if they occur in response to other alterations in postsurgical physiological responses.

1.2 Chemosensory signaling pathways in the gut

RYGB surgery is an invasive technique but mimicking the sustained and enhanced release of GLP-1 and PYY and reducing postprandial ghrelin levels through combination therapy might be a valuable, non-invasive alternative for bariatric surgery. The magnitude of postprandial gut hormone release depends on the caloric content and macronutrient composition of the meal. Several taste receptors (sweet, umami, bitter, fatty acid) and the taste receptor coupled G-protein, α-gustducin, are not only present on taste buds of the tongue but also on enteroendocrine cells (EECs) and may tune gut hormone release in response to a meal. However, the physiological role of taste receptors on EECs has not yet been fully elucidated. In this PhD manuscript we mainly focused on the carbohydrate and SCFA sensing mechanisms of ghrelin secreting X/A cells and GLP-1 and PYY containing L-cells as targets for new anti-obesity treatment strategies. The next part therefore only focuses on the chemosensing pathways of carbohydrates and SCFAs in the gut.

1.2.1 Chemosensing on the tongue

Animals and humans can perceive five basic tastes; sweet, salty, bitter, sour and umami. Because fat can be recognized based on its texture, it is still a matter of debate whether it should be considered as a basic taste. The taste quality of molecules plays an important role in their perception as nutritional or harmful compounds. Sweet taste is present in energy-rich food and indicates a carbohydrate source of calories. Umami taste is an appetitive taste elicited by L-glutamate present in protein-rich food. Salty taste regulates the intake of minerals, required for electrolyte homeostasis and water balance. Bitter, present in many poisonous substances elicits an aversive reaction in humans. Ion channels play a role in the detection of sour taste, which signals the presence of ingested acids and aims to prevent the intake of unripe fruits or spoiled food (Chaudhari and Roper, 2010). Besides dietary guidance, the thought, sight and smell of nutrients stimulate a cephalic phase response (Smeets et al., 2010).

The taste (gustatory) system enables us to sense these tastants through taste receptors on taste buds. A taste receptor can be defined as a receptor protein that recognizes ligands belonging to one of the six taste modalities: salty, sweet, bitter, sour, umami and fat. The best characterized taste receptors are G protein-coupled receptors (GPCRs) and are divided into two classes; the taste receptor family type 1 (TAS1R) and the taste receptor family type 2 (TAS2R). TAS1R1 and TAS1R2 are the first receptors of the TAS1R family that have been identified via a large screening of taste receptor cells (Hoon et al., 1999). TAS1R3 was reported to be co-expressed with TAS1R1 and TAS1R2 and TAS1R1-TAS1R3 and TAS1R2-TAS1R3 can function as an umami and sweet taste receptor, respectively (Nelson et al., 2001, Li et al., 2002, Nelson et al., 2002). Amino acids are not only sensed through TAS1R1-TAS1R3, but also by the calcium sensing receptor, G protein-coupled receptor class C group 6 member A and the metabotropic receptors. The lysophosphatidic acid receptor 5 is considered as a di-and tri-peptide receptor. The family of TAS2Rs has been discovered through

genome screening techniques. In humans, the TAS2R family consists of 25 functional bitter taste receptors (Adler et al., 2000, Chandrashekar et al., 2000, Matsunami et al., 2000). Furthermore, free fatty acid receptors (FFAR) have been identified that can be activated by long- (FFAR1, FFAR4), medium- (FFAR1, FFAR4) (Briscoe et al., 2003, Fredriksson et al., 2003) and SCFAs (FFAR2, FFAR3) (Le Poul et al., 2003, Brown et al., 2003). Sour and sodium are not detected through GPCRs but through ion channels (Huang et al., 2006).

The sensory responses to sweet, umami and bitter are initiated by the binding of the tastant to these GPCRs. These GPCRs couple to downstream signaling effectors that include Gβγ activation of phospholipase Cβ2 ($PLC\beta_2$), 1,4,5-inositol trisphosphate (IP_3) mediated Ca^{2+} release from intracellular stores and Ca^{2+}-dependent activation of the monovalent selective cation channel, the transient receptor potential cation channel subfamily M member 5 (TRPM5) which leads to membrane depolarization and the release of ATP as a transmitter to activate gustatory afferents (Lindemann, 2001, Margolskee, 2002). The function of the α-gustducin subunit in this cascade is not yet fully elucidated. It has been proposed that the basal activity of α-gustducin keeps the cAMP levels in the cell low by phosphodiesterase activation (Yan et al., 2001, McLaughlin et al., 1992, Ruiz-Avila et al., 2001, Ruiz-Avila et al., 1995). These low cAMP levels inhibit protein kinase A activity, which otherwise seems to inhibit $PLC\beta_2$ and IP_3-mediated Ca^{2+}-responses to tastants. Mice that lack α-gustducin are deficient for sweet, umami and bitter (Wong et al., 1996, He et al., 2004), but are not completely insensitive to these stimuli, indicating that alternative signaling pathways may also play a role. For instance, some taste receptors couple to other G-protein subunits of the $G\alpha_i$-subfamily, including α-transducin (Ruiz-Avila et al., 1995, He et al., 2004, Ueda et al., 2003, Sainz et al., 2007, Ozeck et al., 2004).

1.2.2 Chemosensing in the gut

The gastrointestinal (GI) system has a surface membrane area of ~260-300m^2 according to textbooks, although recent analyses using light and electron microscopy report the surface area to be about ~32m^2 of which about 2m^2 refers to the large intestine (Helander and Fandriks, 2014). With this large surface area, the intestine continuously monitors the composition of the luminal content. The idea that the gastrointestinal system is a sensory organ was first suggested by Bayliss and Starling who discovered the gut hormone secretin, and showed that it was released by luminal acid (Bayliss and Starling, 1902). Later it has been shown that the intestine is endowed with a range of cells, such as enterocytes, and EECs that sense nutrients directly and send signals from the epithelium to the vagal sensory afferents in the lamina propria as depicted in Figure 1.4.

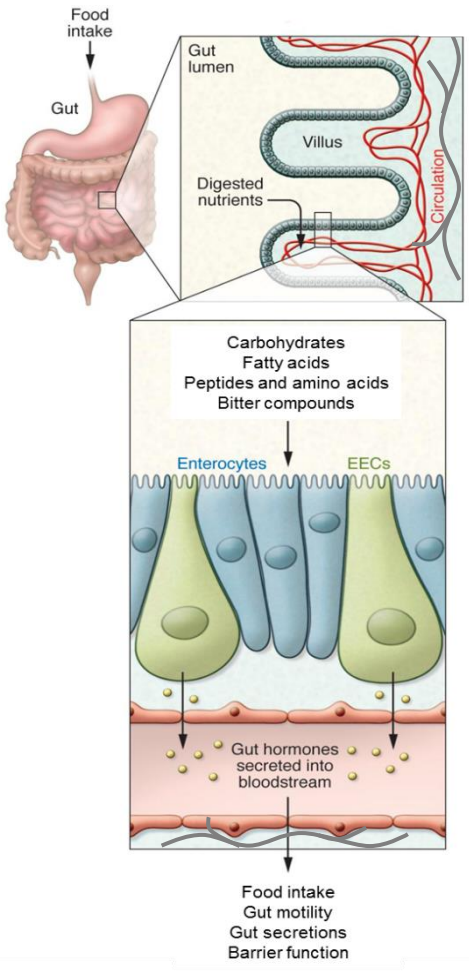


Figure 1.4 Chemosensing in the gut. Ingested food is digested into its nutrient metabolites in the lumen of the small intestine. The intestinal epithelium is arranged in absorptive enterocytes and secretory EECs. Nutrients stimulate EECs in the gut and trigger the secretion of gut hormones, which partially trigger the body's postprandial response. (Adapted from Psichas, J Clin Invest., 2015).

1.2.2.1 Enteroendocrine cells

EECs are specialized epithelial cells that constitute the largest endocrine organ of the body despite the fact that they occupy less than 1% of the epithelial cell population. At least 15 types of EECs have been described, capable of secreting over 20 peptide hormones that influence processes including gut motility, gastric acid secretion, and energy intake. It was previously thought that EECs could be separated into discrete classes of cells with specific secretory profiles; G-cells produce gastrin, P/D1 or X/A-cells ghrelin, D-cells somatostatin, I-cells CCK, entero-chromaffin cells produce serotonin, K-cells GIP and L-cells produce glucagon-like peptides and PYY. However, recent studies using fluorescence-activated cell sorting analysis and microarray expression profiling suggested that most EECs contain more than just one hormone (Egerod et al., 2012, Habib et al., 2012). The hormones released by EECs can act locally, on nerve endings, on other cells, or on organs at remote sites including pancreatic islets and the CNS.

Two groups of EECs can be distinguished according to their shape and localization (Solcia et al., 2000). The first type are the 'open type cells' with microvilli extending into the intestinal lumen which enables them to sense the luminal content directly. After stimulation by the luminal content they can release gut hormones. In contrast to the 'open type cells', the 'closed type cells', do not reach the epithelial surface and can only indirectly be affected by the luminal content through neural and humoral pathways.

Taste receptors of the types previously characterized in the oral cavity were first reported in the intestine in 2001 (Wu et al., 2002). Symonds *et al.* investigated the relative distribution of the transcripts of taste receptors throughout the mouse and human gut. Most taste receptors were expressed throughout the mouse GI tract from stomach to colon, with the exception of TAS1R2 which was only detected in the small intestine. Furthermore, several amino acid receptors and the SCFA receptors were more highly expressed distally (Symonds et al., 2015). EECs can sense the luminal content via activation of these different taste receptors on their membrane. The presence of these nutrient receptors on EECs has been reviewed by Depoortere *et al*. (Depoortere, 2014) and is depicted in Figure 1.5.

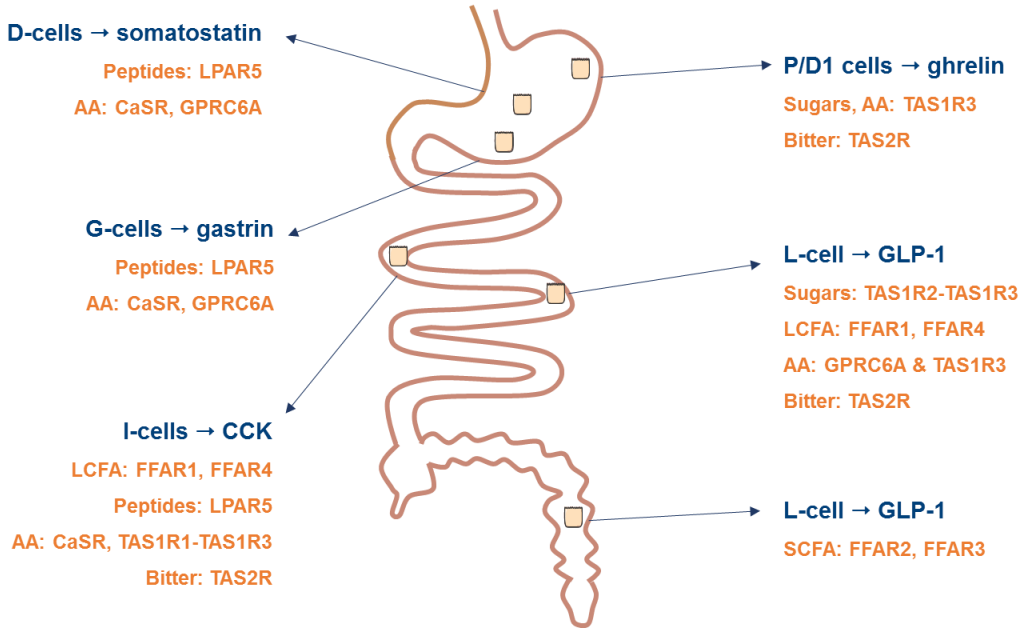


Figure 1.5 Nutrient sensing in the gut. Overview of the expression of taste receptors in different types of EECs along the gut that control the release of gut hormones in response to nutrients. (Adapted from Depoortere, Gut, 2014). Abbreviations: AA, amino acids; CaSR, calcium sensing receptor; FFAR1, free fatty acid receptor 1; FFAR2, free fatty acid receptor 2; FFAR3, fatty acid receptor 3; GPRC6A, G-protein coupled receptor family C group 6 member A; LCFA, long-chain fatty acids; LPAR5, Lysophosphatidic acid receptor 5; TAS1R1, taste receptor type 1 member 1; TAS1R2, taste receptor type 1 member 2; TAS1R3, taste receptor type 1 member 3; TAS2R, taste receptor type 2.

Not only the taste receptors, but also several signaling molecules that play an important role in the taste signaling pathway on the tongue are expressed by EECs. For instance, α-gustducin is expressed by the X/A and L-cells (McLaughlin et al., 1992, Jang et al., 2007, Janssen et al., 2011) (Rozengurt et al., 2006). Furthermore, glucose- and SCFA induced GLP1 release and bitter-induced ghrelin release is blunted in α-gustducin knockout (α-gust$^{-/-}$) mice (Jang et al., 2007, Li et al., 2013, Janssen et al., 2011).

In contrast to α-gustducin, TRPM5 may not play a role in the direct sensing of tastants by EECs. A study in mice showed that TRPM5-positive cells in the mouse duodenum, jejunum and ileum do not contain secretory granules characterized by the presence of chromogranin A and/or B (Kokrashvili et al., 2009b). Moreover, they also did not express some of the hormones known to be present in intestinal enteroendocrine and enterochromaffin cells such as GLP-1, secretin, somatostatin, and serotonin. The TRPM5-positive cells however did co-express the opioid peptides beta-endorphin, Met-enkephalin and uroguanylin (Kokrashvili et al., 2009b) and were later identified as tuft cells that sense the luminal content and signal to neighboring EECs to tune gut hormone release to the luminal content (Kaske et al., 2007, Eberle et al., 2013).

Nevertheless studies in STC-1 cells did show that the foodborne contaminant, vomitoxin, elicits Ca^{2+} dependent secretion of CCK and GLP-1 via activation of TRPM5, by using the TRPM5 channel inhibitor triphenylphosphine oxide (Zhou and Pestka, 2015). Furthermore, linoleic acid induced CCK secretion in STC-1 cells was greatly diminished after siRNA knockdown of TRPM5 (Shah et al., 2012). However, microarray analysis of primary mouse L-cells showed very low levels of TRPM5 but high expression levels of TRPA1, TRPC1, TRPC3 and TRPM7 (Emery et al., 2015). Furthermore, in primary intestinal cultures GLP-1 secretory responses to GW9508 (a FFAR1 agonist) were impaired by an inhibitor of the TRPC3 channel, Pyr3 (Gribble et al., 2016). These results suggest that TRPM5 plays a minor or no role in the direct sensing of EECs.

1.2.2.1.1 Gastric X/A cells

Ghrelin cells or X/A cells are EECs that produce the orexigenic hormone ghrelin and are mostly located in the oxyntic gland of the stomach and to a lesser extent in the small and large intestine (Date et al., 2000). Ghrelin cells exist as open and closed type cells. The number of the open-type cells gradually increases in the direction from the stomach to the lower gastrointestinal tract (Sakata et al., 2002).

Sweet sensing mechanism of the X/A cell

Foster-Shubert *et al.* concluded that the postprandial decline in plasma ghrelin levels is dependent on the macronutrient composition of the meal: Ingestion of carbohydrates decreases plasma ghrelin levels more than the ingestion of proteins or lipids (Foster-Schubert et al., 2008). This suggests that the ghrelin cell contains the machinery to sense nutrients. Indeed, TAS1R3, a component of both the sweet and umami taste receptor, has been localized in ghrelin containing X/A cells and in brush cells in the stomach (Hass et al., 2010), although the TAS1R2 subunit was reported to have a very low (Koyama et al., 2016) or no (Iwatsuki et al., 2010) gastric expression. In healthy patients, artificial sweeteners that can bind the TAS1R2-TAS1R3 receptor did not elicit differences in plasma ghrelin levels (Steinert et al., 2011a, Brown et al., 2011). Therefore the TAS1R3 subunit may indicate the presence of the umami taste receptor instead. Indeed, amino acids have been shown to affect ghrelin release from a stomach-derived cell line and gastric segments (Vancleef et al., 2015).
Previous studies also suggested that K_{ATP} channels are involved in the glucose-dependent ghrelin secretion in MGN3-1 cells (a gastric ghrelinoma cell line) (Oya et al., 2015). However, tolbutamide (a potassium channel blocker) and diazoxide (a potassium channel activator) neither enhanced nor inhibited glucose-induced ghrelin secretion in primary cultures of gastric mucosal cells (Sakata et al., 2012). Therefore the sweet sensing mechanism of the ghrelin cell remains to be elucidated.

SCFA sensing mechanism of the X/A cell

While most digestible carbohydrates will be transported out of lumen in the proximal intestine, non-digestible carbohydrates can reach the distal intestine where some will be fermented by the gut microbiota. The produced fermentation products will include the SCFAs. SCFAs are fatty acids with a carbon chain length of 6 carbons or less. The major SCFA products generated by the microbiome are acetate (C2), propionate (C3) and butyrate (C4). These microbial fermentation products can activate the SCFA receptors FFAR2 and FFAR3. FFAR2 is activated by all three SCFAs and most potently by propionate and acetate (Le Poul et al., 2003), while FFAR3 has a higher affinity for propionate and butyrate than for acetate (Brown et al., 2003). Besides their difference in ligand affinity FFAR2 and FFAR3 also differ in their signaling pathway. Both are GPCRs that are coupled to $G\alpha_{i/o}$, the pertussis toxin-sensitive G protein. In addition, FFAR2 can also couple to the pertussis toxin-insensitive $G\alpha_q$ (Brown et al., 2003). The signaling pathway of FFAR2 and FFAR3 is depicted in Figure 1.6.

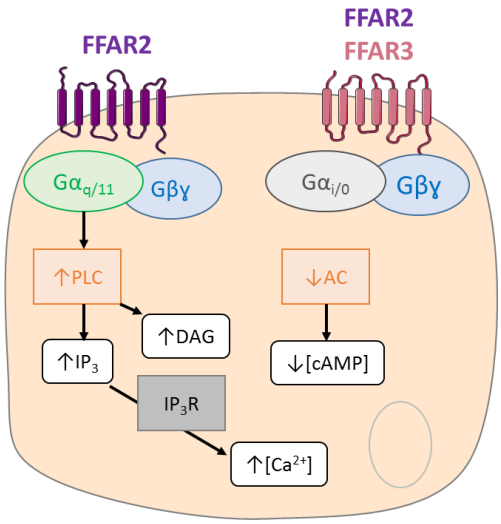


Figure 1.6 FFAR2/3 signaling pathways. Overview of the possible signaling pathways of the FFAR2 and FFAR3 receptos. (Based on Brown, J Biol Chem, 2003). Abbreviations: AC, adenylate cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; FFAR, free fatty acid receptor; IP3, inositol triphosphate; IP3R, inositol triphosphate receptor; PLC, phospholipase C.

The gastric X/A cells, harvested from ghrelin-hrGFP reporter mice, colocalize with both FFAR2 and FFAR3, indicating that SCFAs might be able to bind these receptors to regulate ghrelin secretion (Engelstoft et al., 2013). The hypothesis that the decreased ghrelin secretion after SCFA supplementation is mostly FFAR2 mediated is strengthened by several observations; 1) propionate induced ghrelin release was markedly attenuated in ghrelin cells from FFAR2$^{-/-}$, but not FFAR3$^{-/-}$mice, 2) acetate and propionate decreased ghrelin secretion from primary gastric mucosal cells with similar efficacy, although acetate is a poor ligand for FFAR3, 3) selective agonists and antagonists for FFAR2 and FFAR3 showed a predominant role for FFAR2 and 4) the expression level and degree of enrichment after fluorescence-activated cell sorting (about 20-fold in ghrelin positive compared to ghrelin negative cells) of FFAR2 was much greater compared to FFAR3 (Engelstoft et al., 2013). In contrast, Koyama *et al.* observed that neither acetate nor propionate affected ghrelin secretion from a mouse ghrelinoma cell line (Koyama et al., 2016).

1.2.2.1.2 Intestinal L-cells

Mature L-cells are commonly defined as EECs that express the preproglucagon gene. Posttranslational processing of preproglucagon is tissue-specific, and hence yields different bioactive proteins whether it is processed in the pancreas (glucagon, glicentin-related pancreatic polypeptide, major proglucagon fragment) or gut/brain (GLP-1, GLP-2, oxyntomodulin, glicentin) (Holst et al., 1994, Larsen et al., 1997a).

Spectrum of L-cells

L-cells co-secrete distinct peptides depending on their location. L-cells in the upper small intestine demonstrate co-localization with GIP (Habib et al., 2012). L-cells that co-express GLP-1 and PYY are located along much of the length of the gastro-intestinal epithelium, starting at the proximal jejunum and increasing in density towards the colon (Eissele et al., 1992). The GLP-1 and PYY co-expressing L-cells are typically considered to be involved in the regulation of energy homeostasis, in addition to other functions.

GLP-1 and PYY exhibit a two-phase release profile after a meal. The initial rapid rise in GLP-1 may partially represent release from L-cells in the upper small intestine, although it is thought that most

of this first phase response for GLP-1 and that of PYY is mediated via a neural reflex or a circulating factor. The arrival of food in the large intestine is thought to drive the second phase of the release of GLP-1 and PYY into the circulation, by activation of specific nutrient receptors and other cellular machinery present on the L- cell.

Sweet sensing mechanism of the L-cells

Glucose is a robust stimulant of incretin release. Three possible glucose sensors are proposed by which the L-cell might couple glucose detection to GLP-1 secretion: the sweet taste receptor (TAS1R2-TAS1R3), the sodium coupled glucose transporter (SGLT1) and ATP-sensitive K^+ (K_{ATP}) channels (Figure 1.7). The sweet taste receptor and glucose transporter are predominantly present in the small intestine (Steinert et al., 2011a), while K_{ATP} channels have been proposed to play a role in colonic glucose sensing (Reimann et al., 2008). Activation of the sweet taste receptor, coupled to the gustatory G-protein, increases calcium from intracellular stores, leading to gut hormone release. In contrast, SGLT1 and K_{ATP} channels both couple electrical current to gut hormone release.

The hypothesis that TAS1R2-TAS1R3 coupled to α-gustducin may function as a glucose sensor of the L-cell has been supported by different lines of evidence. Firstly, TAS1R2, TAS1R3 and α-gustducin are present in rodent and human L-cells of the small intestine (Margolskee et al., 2007, Jang et al., 2007). Secondly, several sweet taste receptor agonists, such as the carbohydrates glucose, fructose and the artificial sweetener sucralose were able to elicit GLP-1 secretion from mouse (GLUTag) and human (NCI-H716) enteroendocrine lines and mouse jejunal and ileal explants (Jang et al., 2007). Thirdly, this glucose-induced GLP-1 secretion, was almost or completely impaired in knockout animals for α-gustducin or TAS1R3 (Jang et al., 2007) or in the presence of a sweet taste receptor antagonist; lactisole in Hutu-80 cells (Ohtsu et al., 2014). Finally, human studies showed the involvement of the sweet taste receptor in glucose-induced GLP-1 and PYY secretion, using lactisole (Gerspach et al., 2011, Steinert et al., 2011b).

However, despite promising *in vitro* results most *in vivo* studies show no effect of artificial sweeteners on GLP-1 release in healthy volunteers (Ford et al., 2011, Wu et al., 2012, Ma et al., 2009). These results question the physiological relevance of the sweet taste receptor complex in gut hormone secretion.

A second proposed glucose sensor is Na^+-dependent glucose uptake by the glucose transporter SGLT1 which is colocalized with intestinal L-cells (Reimann et al., 2008). The small transporter-associated currents generated by SGLT1, can drive membrane depolarization, triggering electrical activity, resulting in voltage-gated calcium entry and peptide release (Gribble et al., 2003). This idea is supported by the fact that glucose-dependent GLP-1 and GIP secretion *in vitro*, are prevented in the presence of SGLT1 antagonists in GLUTag cells and primary cell cultures (Parker et al., 2012). Furthermore $SGLT1^{-/-}$ mice have impaired GIP and GLP-1 release after glucose gavage (Gorboulev et al., 2011). Reimann *et al.* further showed that the sweet taste receptor is not involved in these

effects since GLP-1 release was induced at glucose concentrations too low to activate the sweet taste receptor complex (Gorboulev et al., 2011, Reimann et al., 2008).

SGLT1 and GLUT2 expression was upregulated in brush-border membrane vesicles from the intestine of wild type mice, but not of TAS1R3$^{-/-}$ and α-gustducin$^{-/-}$ mice, that were fed a high carbohydrate diet (70% sucrose) for 2 weeks. This suggests a relationship between the activation of the TAS1R2-TAS1R3 in the L-cells and the expression of glucose transporters in enterocytes (Margolskee et al., 2007).

A third possible glucose-sensor is the ATP-sensitive K^+ channel. L-cells and K-cells express glucokinase and K_{ATP} channel subunits (Nielsen et al., 2007). This machinery can couple glucose metabolism, and subsequent ATP generation, to membrane depolarization resulting in voltage-gated calcium entry and peptide release (Reimann and Gribble, 2002). The reduced K^+-efflux lowers the threshold for action potentials, opening voltage dependent Ca^{2+} channels which may result in higher GLP-1 release (Reimann and Gribble, 2002). The K_{ATP} channel blocker tolbutamide increased GLP-1 secretion from GLUTag cells and from isolated L-cells from upper small intestine and colon (Reimann and Gribble, 2002, Reimann et al., 2008). However, K_{ATP} channel blockers did not affect glucose-induced GLP-1 secretion in humans (Stephens et al., 2011). Therefore metabolism-dependent pathways may not be responsible for glucose induced GLP-1 concentrations (Kuhre et al., 2014). Overall, our current understanding of the importance of the sweet taste receptor, SGLT1 and K_{ATP} channels on EECs remains limited.

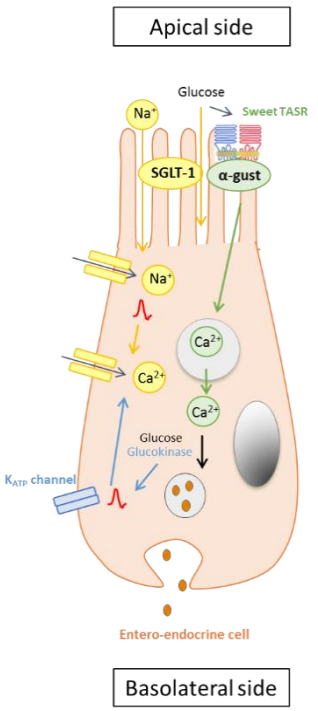


Figure 1.7 The glucose sensing mechanisms of the L-cell. Several glucose sensors have been proposed to regulate glucose-induced GLP-1 release from L-cells. A fist mechanism is sweet taste receptor (TAS1R2-TAS1R3) activation which induces Ca^{2+} release from intracellular stores. A second proposed glucose sensor is Na^+-coupled glucose uptake by SGLT1, which generates small currents that trigger depolarization and voltage-gated Ca^{2+} entry. A third possible candidate is ATP-induced closure of ATP-sensitive (K_{ATP}) channels (induced by glucose metabolism) which trigger voltage gated Ca^{2+} entry. Abbreviations: α-gust, α-gustducin; SGLT1, sodium coupled glucose transporter 1; TASR, taste receptor; K_{ATP} channel, ATP-sensitive K^+ channel.

SCFA sensing mechanism of the L-cell

FFAR2 and FFAR3 are both expressed in the ascending colon and are co-localized with the PYY and GLP-1 secreting endocrine L-cells (Sykaras et al., 2012, Nohr et al., 2013). *In vitro* studies using isolated primary colonic cultures showed that SCFAs stimulate GLP-1 and PYY secretion in a FFAR2 and FFAR3 dependent manner (Tolhurst et al., 2012, Psichas et al., 2015, Karaki et al., 2006). FFAR2$^{-/-}$ mice show lower basal and glucose induced GLP-1 levels (Tolhurst et al., 2012) and reduced portal vein GLP-1 and PYY levels after propionate infusion, indicating that FFAR2 is important for GLP-1 secretion (Psichas et al., 2015). The impaired glucose-induced GLP-1 secretion observed in FFAR2$^{-/-}$

mice was less pronounced in FFAR3$^{-/-}$ mice (Tolhurst et al., 2012). Lin *et al*. confirmed that FFAR3 plays a minor role in butyrate-induced stimulation of GLP-1, and is not required for butyrate- and propionate-dependent induction of GIP (Lin et al., 2012). In contrast, germ-free mice which have no colonic fermentation, display increased GLP-1 levels and delayed gastrointestinal transit (Wichmann et al., 2013). This has been suggested to serve as an adaptive mechanism to inadequate energy availability in the colon. The increased GLP-1 levels would delay transit and hence increase nutrient absorption.

1.2.2.1.3 Opposite regulation of gastric X/A cells and intestinal L-cells by chemoreceptors

Ghrelin and GLP-1 have opposite secretory pattern where ghrelin levels increase before the meal and decrease postprandially and GLP-1 levels increase after a meal. However, their secretory pattern is controlled by similar chemosensors as discussed in the previous paragraphs. Most taste receptors controlling ghrelin secretion are inhibitory while the activation of these taste receptors on L-cells induces stimulatory responses. This dichotomy in function of these taste receptors can be obtained through 1) a combination of the differential expression of several receptors on gastric X/A (no expression of $G\alpha_s$-coupled receptors TGR5 and GPR119) and intestinal L-cells (weak expression of $G\alpha_i$-coupled lactate receptor GPR81), 2) the presence of different receptors for medium-and long chain fatty acids ($G\alpha_i$-coupled FFAR4 on ghrelin cells and the $G\alpha_q$-coupled FFAR1 on the GLP-1 cells) and 3) cell-dependent signaling bias by metabolite receptors that are able to signal through $G\alpha_i$ in the ghrelin cells and conceivably through $G\alpha_q$ in the GLP-1 cells (Engelstoft and Schwartz, 2016).

1.2.2.2 Enterocyte

Enterocytes are the major cell type lining the gut. They express several transporters regulating uptake of sugars, amino acids and fatty acids. On their apical side they contain microvilli to enlarge the luminal contact surface. Enterocytes regulate nutrient uptake and can therefore be seen as absorptive cells which do not possess secretory vesicles.

1.2.2.2.1 Carbohydrate transport

Enterocytes predominantly transport carbohydrates out of the lumen of the small intestine in the form of free glucose and fructose. Polysaccharides are first digested by amylases (present in saliva and pancreatic fluids) to disaccharides, which are digested to monosaccharides by the brush border enzymes; maltase, sucrase-isomaltase, lactase and trehalase. Furthermore, HCl present in the stomach can also catalyze the hydrolysis of sucrose to glucose and fructose. The model for glucose transport is depicted in Figure 1.8. Glucose is transported across the apical membrane via active transport through SGLT1 and can be metabolized intracellularly (Crane, 1965). In a state of low luminal glucose concentrations, the passive glucose transporter GLUT2 can transport glucose from the blood stream into the enterocyte to ensure glucose metabolism, while in a state of high luminal

glucose, GLUT2 will transport the excess of glucose across the basolateral epithelial membrane (Thorens et al., 1988). Strikingly GLUT2$^{-/-}$ animals only show a modest reduction (Roder et al., 2014), or no reduction at all (Stumpel et al., 2001) in peripheral blood glucose levels after a high luminal glucose bolus. These observations suggest the existence of an additional glucose transporter in the basolateral membrane.

High luminal glucose concentrations increase the expression of SGLT1 (Lescale-Matys et al., 1993) which has been hypothesized to mediate the incorporation and upregulation of GLUT2 in the apical membrane (Kellett and Helliwell, 2000). The presence of apical GLUT2 was demonstrated after a glucose bolus in intestinal tissue rings (Gouyon et al., 2003) and isolated intestinal segments (Chaudhry et al., 2012). Furthermore apical GLUT2 has also been shown in *ob/ob* mice, mice on a low-carbohydrate/high-fat diet and in 76% of the biopsies of obese subjects (Ait-Omar et al., 2011). Despite these findings there is some controversy to whether or not GLUT2 can translocate to the apical membrane; 1) lean subjects did not show any apical GLUT2 insertion (Ait-Omar et al., 2011), 2) Röder *et al.* did not observe an increased SGLT1 expression, nor apical GLUT2 insertion after a high oral glucose bolus in mice (Roder et al., 2014), 3) SGLT1$^{-/-}$ mice develop glucose-galactose malabsorption syndrome pointing towards SGLT1 as the predominant transporter (Gorboulev et al., 2011), 4) radiotracer studies of glucose absorption in SGLT1$^{-/-}$ and GLUT2$^{-/-}$ mice revealed a predominant role for SGLT1 in apical glucose transport (Roder et al., 2014) and 5) patients suffering from congenital GLUT2 deficiency (Fanconi-Bickel syndrome) did not display impaired luminal glucose absorption (Santer et al., 2003).

However, Kellet responded to some of these arguments, suggesting that the mice used in the study of Gorboulev *et al*. (Gorboulev et al., 2011) were not optimally handled since the mice were first starved which reduces intestinal GLUT2 apical protein levels (Kellett, 2012). Other studies with GLUT2$^{-/-}$ mice have showed that GLUT2 makes no substantial contribution to the net glucose absorption (Stumpel et al., 2001). Furthermore, D-glucose accumulation in enterocytes is increased in GLUT2$^{-/-}$ mice (Roder et al., 2014). This increase can in part be ascribed to loss of GLUT2 mediated transport activity from the baso-lateral membranes. Therefore evidence on the importance of GLUT2 mediated apical glucose transport remains limited and controversial but suggests that GLUT2 does not play an important role in apical glucose transport.

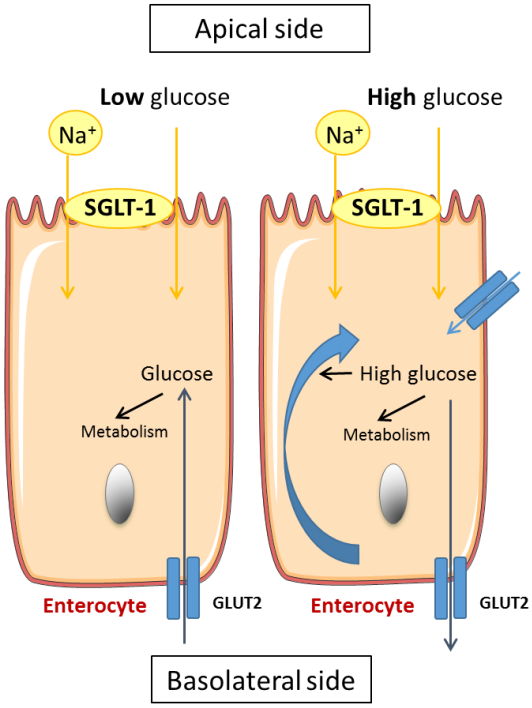


Figure 1.8 The model of intestinal glucose absorption before and after a meal. Before the meal glucose is transported across the apical membrane by SGLT1. The basolateral GLUT2 operates in the opposite direction to supply glucose from the blood and maintain the energy requirements of the cell. At high luminal glucose concentrations initial transport across the apical membrane occurs through SGLT1. This may result in apical insertion of GLUT2. Basolateral GLUT2 will transport the excess of intracellular glucose out of the cell. Abbreviations: GLUT2, glucose transporter 2; SGLT1, sodium coupled glucose transporter 1.

Fructose is transported across the apical membrane via the glucose transporter GLUT5 (Burant et al., 1992), but similarly to glucose it needs GLUT2 to be transported through the basolateral membrane into the blood stream (Thorens et al., 1988).

1.2.2.2.2 SCFA transport

Dietary fibers, like OFS, are carbohydrates which are not digested in the small intestine by human gastrointestinal enzymes. These molecules are fermented to SCFAs by the colonic gut microbiota. Approximately 500-600mmol SCFAs are produced in the human colon per day (Cummings, 1981), whereas fecal SCFA extracts range from 5-30mmol/day (den Besten et al., 2013). This implies that most of the SCFAs are absorbed by the colonocytes (McNeil et al., 1978). A schematic presentation of SCFA transport on the colonocytes is depicted in Figure 1.9. SCFAs can be absorbed by the colonocytes via three different mechanisms; 1) passive diffusion of the protonated SCFA (Hoverstad et al., 1982), 2) exchange with bicarbonate in a 1:1 ratio (Titus and Ahearn, 1988), or 3) active transport of the dissociated SCFAs via a monocarboxylate transporter (MCT). The electrogenic sodium-dependent monocarboxylate transporter 1 (SMCT-1) and the MCTs; MCT-1, MCT-2 and MCT-4, are found in the apical membrane of enterocytes and transport SCFAs (Moschen et al., 2012, Ritzhaupt et al., 1998). Flux measurements of labeled compounds in Xenopus laevis oocytes expressing the isoforms MCT-1, MCT-2 and MCT-4 showed that all these transporters mediate acetate uptake with MCT4 being the weakest acetate transporter (Moschen et al., 2012). MCT-1 can also mediate propionate and butyrate transport, but the bulk of propionate and butyrate transport will be mediated through non-ionic diffusions (Moschen et al., 2012). Furthermore, butyrate uptake by the colonocytes mainly involved MCT-1 and SMCT-1 in rat small intestinal epithelial cells (IEC-6) but not in fetal human colonic epithelial (FHC) cells (Goncalves et al., 2011).

The absorbed SCFAs are not completely metabolized by the colonocytes, but can exit the colonocytes across the basolateral membrane into the blood stream. This basolateral transport into the circulation can be mediated via MCT-4, MCT-5 or transport via the SCFA/HCO_3^- exchanger (Gill et al., 2005). This uptake results in concentrations of SCFAs in the portal blood ranging from 260µM for acetate to 30µM for propionate and butyrate (Bloemen et al., 2009).

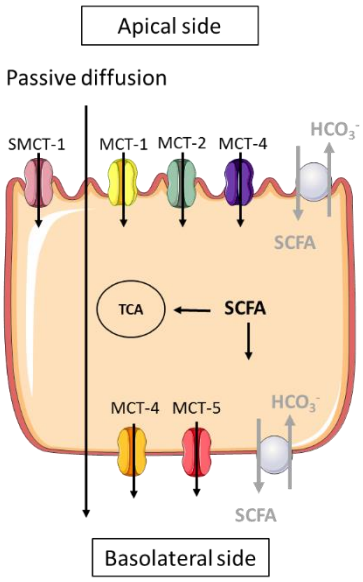


Figure 1.9 The model of intestinal SCFA absorption. SCFAs are absorbed by the enterocytes across the apical membrane via passive diffusion of the protonated SCFA, the SCFA/HCO_3^- exchanger, or active transport of the dissociated SCFAs via SMCT-1, MCT-1, MCT-2 and MCT-4. In the enterocytes, the SCFAs can enter the citric acid circle (TCA) to generate ATP, or they can be transported across the basolateral membrane via MCT-4, MCT-5 or transport via the SCFA/HCO_3^- exchanger. Abbreviations: MCT, monocarboxylate transporter; SMCT, sodium-dependent monocarboxylate transporter; SCFA, short-chain fatty acid; TCA, citric acid circle.

1.2.2.2.3 Brush cells

Brush cells are a specific type of enterocytes with a pear shape and long apical microvilli which overreach those of the enterocytes. They only represent a small portion of the cells in the gastrointestinal tract. Brush cells express gustatory signaling elements, like α-gustducin, transducin and TRPM5, but do not contain secretory granules. Therefore it has been hypothesized that these cells may sense the luminal content and signal the information to nearby EECs (Hass et al., 2007). Furthermore, three recent publications show that infection with parasitic worms such as helminths or protozoa are sensed via brush cells in the gut epithelium. They orchestrate the type 2 cell-mediated immunity by producing IL-25 in mice. Brush cells and goblet cells accumulate during infection in a TRPM5 and gustducin-dependent manner. Up till now it is unclear which infection-induced molecules are sensed by the gustducin coupled taste receptors on brush cells to initiate this signaling cascade (von Moltke et al., 2016, Gerbe et al., 2016, Howitt et al., 2016).

1.2.3 Chemosensing in a setting of obesity

Obesity has been associated with altered meal-induced gut hormone release. Obese and overweight patients reported attenuated postprandial GLP-1 (Adam and Westerterp-Plantenga, 2005, Verdich et al., 2001) and PYY levels (Brownley et al., 2010, Cahill et al., 2011, le Roux et al., 2006), while fasting and postprandial plasma ghrelin levels were reported to be lower in obese compared to normal-weight individuals (Tschop et al., 2001).

Furthermore, changes in circulating peptides during obesity are linked to a change in their receptor expression levels in vagal afferent neurons (Kentish et al., 2013). A HFD increased the expression of the ghrelin and the CCK receptor (GHSR and CCK1R) compared to rats on a normal chow diet (Paulino et al., 2009). Furthermore, the presence of ghrelin in cultured nodose ganglia from lean mice increased CCK1R expression and decreased GHSR expression, while the effect of ghrelin on the expression of these receptors on nodose ganglia from HFD-fed mice was reversed (Kentish et al., 2013). Therefore, obesity does not only alter circulating gut hormone levels and their vagal receptor expression but also disrupts the inter-regulation of appetite regulatory receptors in vagal afferents (Kentish et al., 2013).

The altered gut hormone levels in a setting of obesity coincide with altered expression levels of chemosensory elements on the tongue and in the GI tract. On the tongue diet-induced obesity decreased the detection of some taste stimuli through decreased taste receptor signaling (Maliphol et al., 2013). In gastric tissue, qPCR analyses revealed differences in mRNA expression levels for GPR120, α-gustducin, PLCβ2, TRPM5 and TAS1R3 in obese patients (Widmayer et al., 2012). EECs in the colon of overweight and obese patients showed an upregulation of the bitter taste receptor TAS2R38 (Latorre et al., 2016).

Furthermore, a human genetics study revealed an association between the genetic risk of metabolic syndrome and polymorphisms coding for α-gustducin (Farook et al., 2012). These initial findings led to the hypothesis that the alteration in gut hormone levels and chemosensory elements may be linked and may mediate functional responses occurring during an altered energy balance.

Chapter 2

RESEARCH OBJECTIVES

2 RESEARCH OBJECTIVES

Before the ingestion of a meal, food is sensed by taste receptors on the taste buds of the tongue which transmit signals via sensory afferents to inform the brain about the macronutrient composition of the meal. Sweet and umami are detected by heterodimers of 3 subtypes from the taste receptor family type 1 (TAS1R), bitter by 25 subtypes of the taste receptor family type 2 (TAS2R) and short, medium and long chain fatty acids by 4 subtypes of the free fatty acid receptors (FFAR). The brain will respond accordingly to the transmitted taste signals with learned anticipatory responses.

After the ingestion of a meal, taste receptors in the gut sense nutrients to elicit motor and secretory responses to assimilate the ingested macronutrients. As such, taste receptors on EECs coordinate the release of gut hormones (ghrelin, GLP-1, PYY) involved in appetite regulation.

Evidence suggests that extra-oral taste receptors on EECs play an important role in detecting changes in the intraluminal content associated with a HFD and contribute to the dysregulated gut hormone levels and disturbed appetite signaling in obese patients. For example, a human genetics study revealed an association between the genetic risk of metabolic syndrome and polymorphisms coding for α-gustducin, a gustatory G-protein coupled to several taste receptors (sweet, fatty acid, umami, bitter) (Farook et al., 2012). Furthermore, in obese patients the mRNA expression levels of several chemosensory elements are altered in the stomach and bitter taste receptor expression is upregulated in EECs of the colon (Widmayer et al., 2012, Latorre et al., 2016). These initial findings further strengthen the hypothesis that chemosensory elements may mediate functional responses occurring during an altered energy balance.

We investigated whether current treatment strategies for obesity such as the usage of functional foods (sucralose, oligofructose) or gastric bypass surgery interfere with the molecular events associated with intraluminal chemosensing. Since α-gustducin is coupled to several taste receptors (Jang et al., 2007, Janssen et al., 2011, Li et al., 2013), α-gustducin$^{-/-}$ mice were used in all our studies to elucidate the role of gut chemosensing in the applied treatments.

In the first aim of this study, we investigated whether targeting the α-gustducin-mediated sweet taste receptor signaling pathway has an acute effect on the release of the hunger hormone ghrelin, using carbohydrates, artificial sweeteners or prebiotic carbohydrates

In the second aim we studied whether long-term targeting of taste receptors with sweeteners in diet-induced obese mice has beneficial effects on gut hormone release and hence on glucose –and energy homeostasis but also on other events such as the “leaky gut” associated with obesity.

In the last aim we investigated whether the nutrient overexposure or underexposure that occurs in some regions of the gut after Roux-en-Y gastric bypass surgery affects the nutrient sensing mechanisms and hence contributes to the restoration of the gut hormone balance and body weight regulation after bariatric surgery.

The general hypothesis and research objectives of this PhD manuscript is depicted in Figure 2.1.

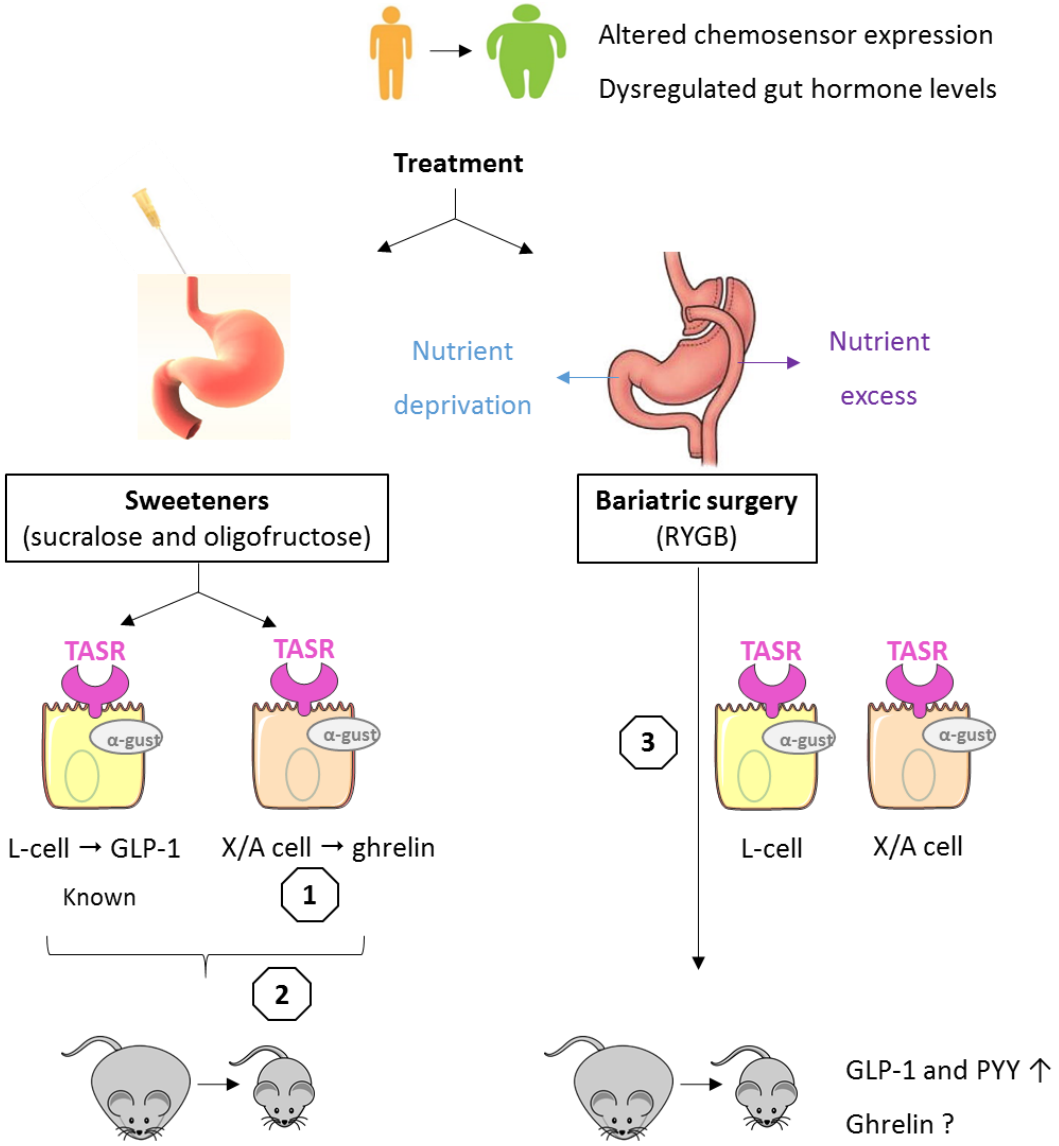


Figure 2.1 Research objectives of the thesis manuscript. Abbreviations: RYGB, Roux-en-Y gastric bypass surgery; TASR, taste receptor.

Aim 1. Unraveling the sweetener sensing mechanisms of the ghrelin cell

Sweet taste receptors (TAS1R2+TAS1R3), coupled to the gustatory G protein, α-gustducin, among others, are involved in the glucose-dependent secretion of the gut satiety peptide, GLP-1 from the L-cells (Jang et al., 2007, Gerspach et al., 2011, Steinert et al., 2011b). Recent studies, including from our lab, showed that the ghrelin cell also expresses TAS1R3 and gustatory G-proteins (Hass et al., 2010, Janssen et al., 2011), but their physiological role has not yet been elucidated.

We investigated whether the α-gustducin-mediated sweet taste receptor signaling pathway is involved in the effect of carbohydrates and sweeteners on ghrelin release in three different models;

1) a ghrelinoma cell line, 2) *ex vivo* preparations from gastric and jejunal segments from wild type (WT) and α-gustducin (α-gust$^{-/-}$) mice and 3) *in vivo* after acute administration of carbohydrates or sweeteners in WT and α-gust$^{-/-}$ mice.

Aim 2. Exploring the role of gustducin-mediated gut hormone release in the effect of sweeteners (oligofructose and sucralose) on high-fat diet induced body weight gain

Artificial sweeteners, such as sucralose, are non-caloric but might not be metabolically inert. Furthermore, the prebiotic sweetener oligofructose (OFS), has been proposed as a functional food ingredient that beneficially affects gut microbiota composition, gut permeability and modulates the secretion of gut hormones to prevent obesity and diet-induced T2DM in rodents (Cani et al., 2004b, Cluny et al., 2015, Cani et al., 2009). Both OFS and sucralose can activate the sweet taste receptor. In addition, colonic fermentation of OFS by the gut microbiota will increase the production of short-chain fatty acids (SCFAs) which can activate FFAR2 and FFAR3 on EECs.

We investigated whether long-term gavage of equisweet concentrations of a prebiotic (oligofructose) or an artificial (sucralose) sweetener could prevent the induction of diet-induced obesity and T2DM by altering gut hormone release through interaction with extra-oral taste receptors coupled to α-gustducin. The underlying pathways were investigated with a specific focus on the effect of the sweeteners on sweet-and fatty acid taste receptor expression along the gut, the release of gut hormones and possible intestinal adaptations such as changes in gut morphology and gut permeability.

Aim 3. Exploring the role of nutrient sensing in the metabolic changes after Roux-en-Y gastric bypass (RYGB) surgery

After RYGB surgery food passes directly from a small stomach pouch into the distal part of the small intestine. Due to this rerouting, gastric acid production is decreased while bile acids and pancreatic enzymes are secreted in the excluded duodenum and reach the undigested food in the distal part of the jejunum. To adapt to the new digestive route we hypothesized that RYGB surgery has a major impact on the extra-oral taste receptors on EECs that control the pattern of gut hormone secretion both in the foregut and distal gut.

We investigated the role of extra-oral gustducin-mediated taste receptor signaling in the altered gut hormone secretion, favoring sustained improvement of glucose -and energy homeostasis after RYGB surgery in obese WT and α-gust$^{-/-}$ mice. In addition, we performed an in-depth study on the origin of the altered gut hormone levels and the physiological adaptations along the gut (e.g. gut morphology and gut permeability).

Chapter 3

UNRAVELING THE SWEETENER SENSING MECHANISMS OF THE GHRELIN CELL

The data represented in the following chapter are published in Nutrients:

The sweetener sensing mechanisms of the ghrelin cell

Steensels S., Vancleef L., Depoortere I.

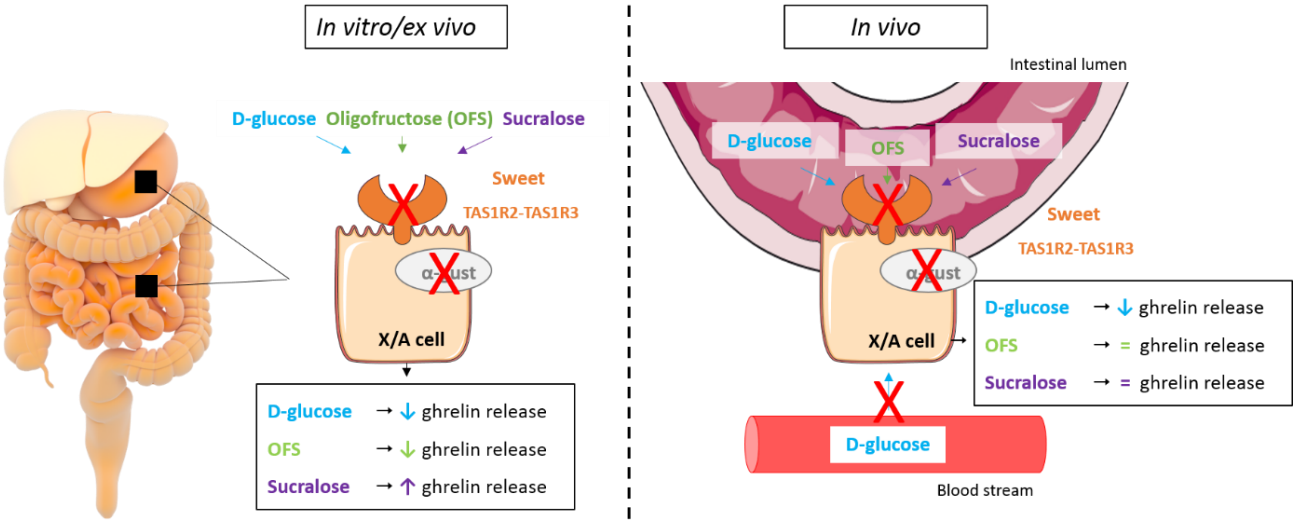
Nutrients **2016**, *8*(12), 795; doi:10.3390/nu8120795.

3 UNRAVELING THE SWEETENER SENSING MECHANISMS OF THE GHRELIN CELL

3.1 Abstract

Carbohydrate administration decreases plasma levels of the 'hunger hormone' ghrelin. The ghrelin cell is co-localized with the sweet taste receptor subunit, TAS1R3, and the gustatory G-protein, gustducin, both involved in the sensing of sweeteners by EECs. This study investigated the role of gustducin mediated sweet taste receptor signaling on ghrelin secretion in a gastric ghrelinoma cell line, tissue segments and mice. The monosaccharide D-glucose and low-intensity sweetener oligofructose decreased (P<0.001) ghrelin secretion while the high-intensity sweetener sucralose increased (P<0.001) ghrelin secretion *in vitro*. These effects were not mediated via the sweet taste receptor or glucose transporters (SGLT1 and GLUT2). The effect of these compounds was mimicked *ex vivo* in gastric and jejunal segments from both wild type (WT) and α-gustducin knockout (α-gust$^{-/-}$) mice. *In vivo*, the sensing of D-glucose was polarized since intragastric but not intravenous administration of D-glucose decreased (P<0.05) ghrelin levels in a α-gustducin-independent manner which involved inhibition of duodenal ghrelin release. In contrast, neither oligofructose nor sucralose affected ghrelin secretion *in vivo*. In conclusion, α-gustducin mediated sweet taste receptor signaling does not play a functional role in the sensing of carbohydrates, low- or high-intensity sweeteners by the ghrelin cell.

3.2 Graphical abstract



3.3 Introduction

Over the past four decades, we have transitioned from a world in which underweight prevalence was more than double that of obesity, to one in which more people are obese than underweight (NCD et al., 2016). This increase in obesity prevalence has been linked to an excessive sugar intake (Medina-RemOn et al., 2016, Bray and Popkin, 2014). Therefore guidelines in healthcare arose, recommending reductions in added sugar intake (WHO, 2015). Sugar replacers, such as HIS (e.g. sucralose), can help reduce the sugar content of meals without affecting its taste. These sweeteners are non-caloric but might not be metabolically inert, since contradictory results have been reported on their impact on energy homeostasis (Roberts, 2015).

Next to these HIS, prebiotic sweeteners such as oligofructose (OFS) have been proposed as functional food ingredients. OFS has a low caloric content (1.7kcal/g) which is derived from its microbial fermentation products, the short-chain fatty acids (SCFAs), which can be used as an energy substrate by the colonocytes. It has a sweetening power of 35% of that of sucrose (table sugar) (Niness, 1999). Furthermore, OFS decreases food intake, fat mass development, and hepatic steatosis in normal and obese rodents (Delzenne et al., 2011b, Woting et al., 2015, Cluny et al., 2015, Anastasovska et al., 2012, Delmee et al., 2006, Cani et al., 2006, Cani et al., 2005b). In humans, contradictory results have been reported with inulin-type fructans on body weight reduction (Liber and Szajewska, 2013).

The hunger hormone ghrelin can stimulate food intake, prevent fatty acid utilization, increase body weight, inhibit glucose-induced insulin release and stimulate gastrointestinal motility (Kojima et al., 1999, Muller et al., 2015, Avau et al., 2013, Verhulst and Depoortere, 2012). Ghrelin needs a posttranslational modification, catalyzed by the enzyme ghrelin-O-acyltransferase (GOAT) to exert its biological activity (Kojima et al., 1999, Gutierrez et al., 2008, Yang et al., 2008). Both GOAT and ghrelin are present in X/A like cells of the gastric oxyntic mucosa.

Plasma ghrelin levels increase before a meal and decrease thereafter to determine the frequency of the meals. Whereas the preprandial rise involves activation of the autonomic nervous system (Zhao et al., 2010), the magnitude of the postprandial decline is dependent on the macronutrient composition of the meal (Foster-Schubert et al., 2008). Whether the latter is mediated via pre- or postabsorptive effects or involves chemosensation by the ghrelin cell is still not clear. However, recent evidence suggests that the ghrelin cell is equipped not only with receptors for neuropeptides but also with receptors for dietary and endogenous metabolites such as amino acids and free fatty acids that can directly regulate ghrelin release (Vancleef et al., 2015, Janssen et al., 2012). Immunohistochemical studies also provided evidence for the presence of gustatory G-proteins (gustducin, transducin) (Janssen et al., 2011) and a subunit of the sweet taste receptor (TAS1R2-TAS1R3) (Hass et al., 2010) on the ghrelin cell but their functional role remains to be elucidated. The sweet taste receptor is broadly tuned to detect glucose and other simple sugars, and is activated by artificial sweeteners (DuBois, 2016). The sweet taste receptor, coupled to gustducin, and the sodium-

dependent glucose cotransporter (SGLT1) act as glucose-sensors of the L-cells in the small intestine (Jang et al., 2007, Gorboulev et al., 2011).

This study aimed to investigate whether α-gustducin mediated sweet taste receptor signaling is involved in the effect of carbohydrates and sweeteners on ghrelin release. A ghrelinoma cell line was used to investigate the *in vitro* effect and the mechanism of action of carbohydrates and sweeteners (sucralose and OFS) on ghrelin release. *Ex vivo* gastric and jejunal segments from wild type (WT) and α-gustducin (α-gust$^{-/-}$) mice were used to determine whether the sweet sensing mechanisms of the ghrelin cell are tissue dependent and involve a sweet taste receptor coupled to the gustatory G-protein, α-gustducin. Finally, the effect of glucose and sweeteners on ghrelin release was investigated *in vivo* in WT and α-gust$^{-/-}$ mice to investigate the role of α-gustducin mediated sweet taste receptor activation and signaling.

3.4 Materials and Methods

3.4.1 Materials

D-glucose was obtained from Merck (Merck, Germany), sucralose, phloridzin and phloretin were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Oligofructose (OFS) was kindly provided by Beneo-Orafti (Beneo-Orafti, Germany) and gurmarin by Prof. L. Briand (Center for Taste and Feeding Behaviour, Dijon, France). The stock solutions of phloretin and phloridzin were made in DMSO and further diluted in Krebs-Ringer buffer with 11mM D-glucose resulting in a final concentration of 0.001% DMSO for 10µM phloretin/phloridzin and 0.002% DMSO for 20µM phloretin,. The ghrelinoma cell line, MGN3-1, was kindly provided by Prof. H. Iwakura (Kyoto University Hospital, Kyoto, Japan).

3.4.2 Mice

Male C57BL/6 WT mice and α-gust$^{-/-}$ mice (kindly provided by Prof. R. Margolskee, Monell Chemical Senses Center, Philadelphia, United States) were kept in the animal facility. All mice were housed (20–22 °C) under a 14-h:10-h light-dark cycle and had *ad libitum* access to food and drinking water. All experimental procedures were approved by the Ethical committee for Animal Experiments of the KU Leuven (P100/2013).

3.4.3 Experimental design

Overnight-fasted mice were either gavaged (150µL) with D-glucose (4g/kg body weight), OFS (5.6g/kg body weight), sucralose (8.95mg/kg body weight) or 0.9% NaCl, or injected intravenously (IV) (150µL) into the tail vene with 1g/kg body weight D-glucose or 0.9% NaCl. In humans sucralose is in general 320-1000 times sweeter than sucrose and sucrose is 1.25-1.43 times more sweet than glucose (Friedman, 1998, Wildman, 2011). This indicates that sucralose is about 1000 times sweeter compared to glucose, resulting in a dose of 8.95 mg/kg for sucralose compared to 4g/kg for glucose. Furthermore, oligofructose is 2 to 3.3 times less sweet than sucrose (Niness, 1999), resulting in a

dose of about 5.6g/kg for OFS. These doses were chosen to be "equisweet" in order to study the effect of the sweeteners after a similar degree of sweet taste receptor activation. However, the "equisweet" doses were based on human studies, although the dose used in human studies does not necessarily apply to mice.

Forty minutes after IV injection or gavage, mice were humanely killed. Blood was collected by cardiac puncture and supplemented with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (4mM) and ethylenediaminetetraacetic acid (1mg/ml). Plasma was acidified (0.1N HCl) and stored at -80°C. The stomach and duodenum were removed and stored for protein extraction.

3.4.4 Ghrelin tissue extraction

Tissue from stomach and duodenum was boiled for 10 minutes followed by homogenization in 3 volumes of water with protease inhibitors (MP Biomedicals, CA) and 9 volumes of 6% acetic acid. After 10 minutes of boiling, the homogenate was centrifuged to collect the supernatant which was diluted and subjected to radioimmunoassay (RIA). Protein levels were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., MA).

3.4.5 Ghrelin release from intestinal segments

Overnight fasted WT and α-gust$^{-/-}$ mice were refed for 2 hours prior to being sacrificed. Segments of the intact corpus of the stomach (0.3x0.3 cm) and jejunum (0.4x1 cm) were dissected and incubated at 37°C in Krebs-Ringer buffer (11mM D-glucose) with the test solutions (D-glucose (200mM), OFS (10%), sucralose (200mM)) for 2 hours. The culture medium was collected, acidified (0.1N HCl) and stored at -80°C. Tissue segments were dried to correct ghrelin release for dry tissue weight of the segment.

3.4.6 Ghrelin release from ghrelinoma cells

MGN3-1 cells were cultured in Dulbecco's modified eagle medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were incubated with D-glucose (11.1-200mM), D-fructose (20-200mM), OFS (0.1-10%) or sucralose (1-200mM) in Krebs-Ringer buffer with 11mM D-glucose for 3 hours. Osmolality was corrected to physiological levels by adapting the concentration of NaCl. The effect of the sweet taste receptor antagonist (30µg/ml gurmarin) (Margolskee et al., 2007), or glucose transporter inhibitors (SGLT1 antagonist; 10µM phloridzin (Parker et al., 2012), GLUT-family antagonist; 10-20µM phloretin (Zou et al., 2014)) was investigated by preincubation of the cells during 30 minutes with the respective inhibitors after which the culture medium was removed and replaced by a combination of the antagonist and the indicated carbohydrate or sweetener for 3 hours. The dose of gurmarin was high enough to block both TAS1R2-TAS1R3 (Margolskee et al., 2007) and the TAS1R3 homodimer since

this dose blocked the umami taste receptor (TAS1R1-TAS1R3) and thus the common subunit of the sweet and umami taste receptor, namely TAS1R3 (Vancleef et al., 2015, Daly et al., 2013). Following the incubation, the supernatant was collected, acidified (0.1N HCl) and stored at -80°C.

3.4.7 Radioimmunoassay (RIA)

Plasma samples and cell/tissue culture supernatants were extracted on a SEP-Pak C18 cartridge (Waters Corporation), vacuum-dried and subjected to ghrelin RIA as previously described (Janssen et al., 2011). For determination of octanoyl ghrelin a rabbit anti-human ghrelin [1-8] antibody was used which does not recognize desoctanoyl ghrelin. Total ghrelin levels were determined using a rabbit anti-human ghrelin [14-28] antibody, which recognizes both octanoyl and desoctanoyl ghrelin.

3.4.8 Quantitative real-time PCR

Total RNA was isolated from MGN3-1 cells and tissue segments from the mouse gastro-intestinal (GI) tract using the RNeasy kit (Qiagen), treated with Turbo DNAfree kit (Ambion) and reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen). The qRT-PCR reaction was performed as described previously, using the Lightcycler 480 (Roche Diagnostics) with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics) (Verhulst et al., 2008), and analyzed using the LightCycler® 480 SW 1.5.1 software (Roche Diagnostics). Results were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used: GAPDH: forward CCCCAATgTgTCCgTCgTg, reverse gCCTgCTTCACCACCTTCT; SGLT1: forward CggAAgAAggCATCTgAgAA, reverse AATCAgCACgAggATgAACA; GLUT2: forward TCTTCACggCTgTCTCTgTg, reverse AATCATCCCggTTAggAACA; TAS1R2: forward gCACCAAgCAAATCgTCTATCC, reverse ATTgCTAATgTAggTCAgCCTCgTC; TAS1R3: forward CAggCAgTTgTgACTCTgTTg, reverse TgCgATgCAgATACCTCgTg.

3.4.9 Statistical analysis

Results are presented as mean ± SEM. The data representing the effect of the test compounds on ghrelin release from intestinal segments and on plasma ghrelin levels and tissue ghrelin content were assessed for normality of distribution. As the data were distributed in a non-normal and/or non-homogeneous manner, log-transformed data were used to examine the main effects of the compounds using a multivariate analysis of variance (MANOVA). An interaction effect between compounds and genotypes was included in the model as well. Post-hoc t-tests with Holm-Sidak correction for multiple testing were applied (SAS Studio University Edition 9.4). Dose-response curves of the test compounds in the MGN3-1 cell line were analyzed using a repeated measures analysis (factors; compound and dose), followed by planned comparisons post-hoc testing and Bonferroni correction (Statistica 12, Statsoft). The effect of the different antagonists on the effect of the test compounds on ghrelin release was analyzed with a two-way ANOVA, followed by planned

comparisons post-hoc testing and Bonferroni correction (factors; compound and antagonists) (Statistica 12, Statsoft). Significance was accepted at the 5% level.

3.5 Results

3.5.1 In vitro studies in the MGN3-1 ghrelinoma cell line

The gastric MGN3-1 cell line shows a strong expression of the TAS1R3 subunit of the sweet taste receptor and the glucose transporters (SGLT1 and GLUT2). The sweet taste receptor subunit TAS1R2 is not detectable in the cell line (Figure 1a).

MGN3-1 cells were incubated with increasing concentrations of D-glucose, oligofructose (OFS) or sucralose and the effect on octanoyl ghrelin release was determined.

D-glucose (200mM) and OFS (10%) induced a significant (P<0.001) decrease in octanoyl ghrelin levels while sucralose (200mM) stimulated (P<0.001) octanoyl ghrelin release. Lower concentrations had no effect (Figure 1b).

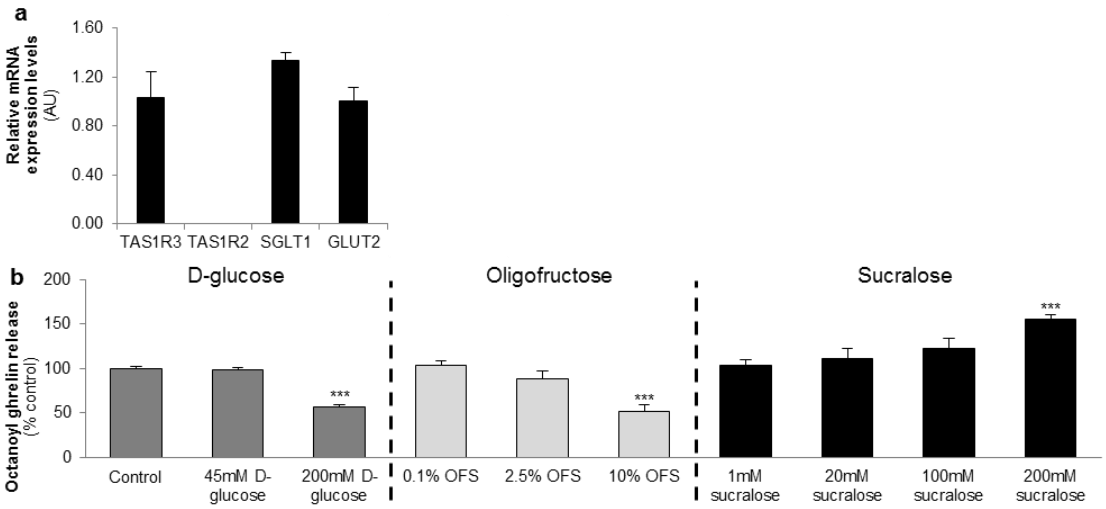


Figure 1. D-glucose and the low-intensity sweetener oligofructose decrease octanoyl ghrelin release while a high-intensity sweetener sucralose increases octanoyl ghrelin secretion from a ghrelinoma cell line. (a) Relative mRNA expression levels of the two subunits of the sweet taste receptor (TAS1R2-TAS1R3), and the glucose (SGLT1, GLUT2) transporters in the ghrelinoma cell line, MGN3-1 (n=3/sensor). (b) Concentration-dependent effect of a 3 h stimulation with D-glucose, oligofructose (OFS) and sucralose on octanoyl ghrelin release (n=9-12). Results (mean ± SEM) are expressed relative to the control stimulation (Krebs buffer containing 11.1mM D-glucose). *** P<0.001 vs. control. AU: arbitrary units

The inhibitory effect of 200mM D-glucose or 10% OFS and the stimulatory effect of 200mM sucralose on octanoyl ghrelin release was not blocked by the sweet taste receptor antagonist gurmarin (30µg/ml), the SGLT1 inhibitor phloridzin (10µM) or the GLUT family inhibitor phloretin (10-20µM) (Figure 2a-e). Phloretin (20µM), but not phloretin (10µM), phloridzin (10µM) or gurmarin (30µg/ml), increased basal ghrelin release with about 60% (P<0.05) (Figure 2e).

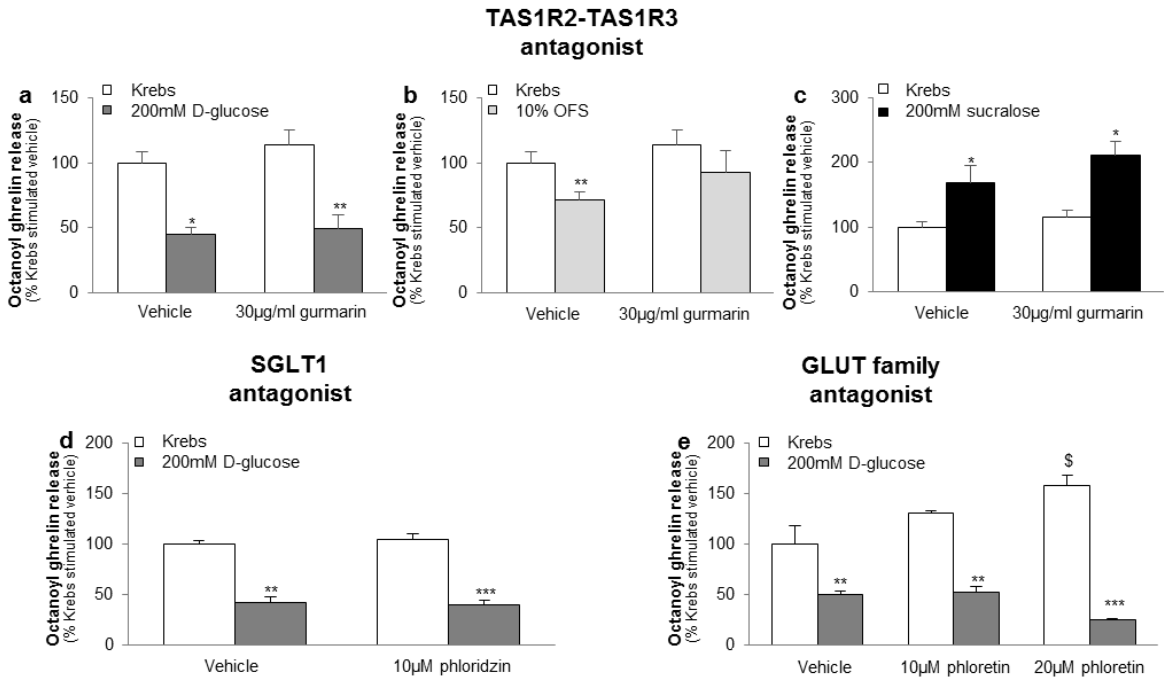


Figure 2. The effect of carbohydrates and sweeteners on octanoyl ghrelin release in the ghrelinoma cell line are not mediated via the sweet taste receptor or the glucose transporters. Effect of preincubation (30 min) of MGN3-1 cells with a (a-c) TAS1R2-TAS1R3 antagonist (gurmarin, 30µg/ml) (n=9), (d) SGLT1 inhibitor (phloridzin, 10µM) (n=9) or (e) GLUT family antagonist (phloretin, 10-20µM) (n=9) or their respective vehicle (Krebs with or without DMSO) on the effect of (a, d, e) 200mM D-glucose, (b) 10% oligofructose (OFS), and (c) 200mM sucralose compared to Krebs buffer on octanoyl ghrelin release in MGN3-1 cells. Results (mean ± SEM) are expressed relative to the control stimulation (Krebs buffer containing 11.1mM D-glucose). * P<0.05, ** P<0.01, *** P<0.001 vs. vehicle, $ P<0.05 vs. vehicle stimulated control.

3.5.2 Ex vivo studies in intestinal segments

The mRNA expression levels of the different glucose sensors were determined in several regions of the GI tract of mice. The TAS1R3 subunit and α-gustducin were expressed throughout the GI tact with a high expression in the stomach and distal GI tract (Figure 3a, c). In contrast, the highest expression levels of the TAS1R2 subunit and the glucose transporters (SGLT1 and GLUT2) were observed in the small intestine (Figure 3b, d, e).

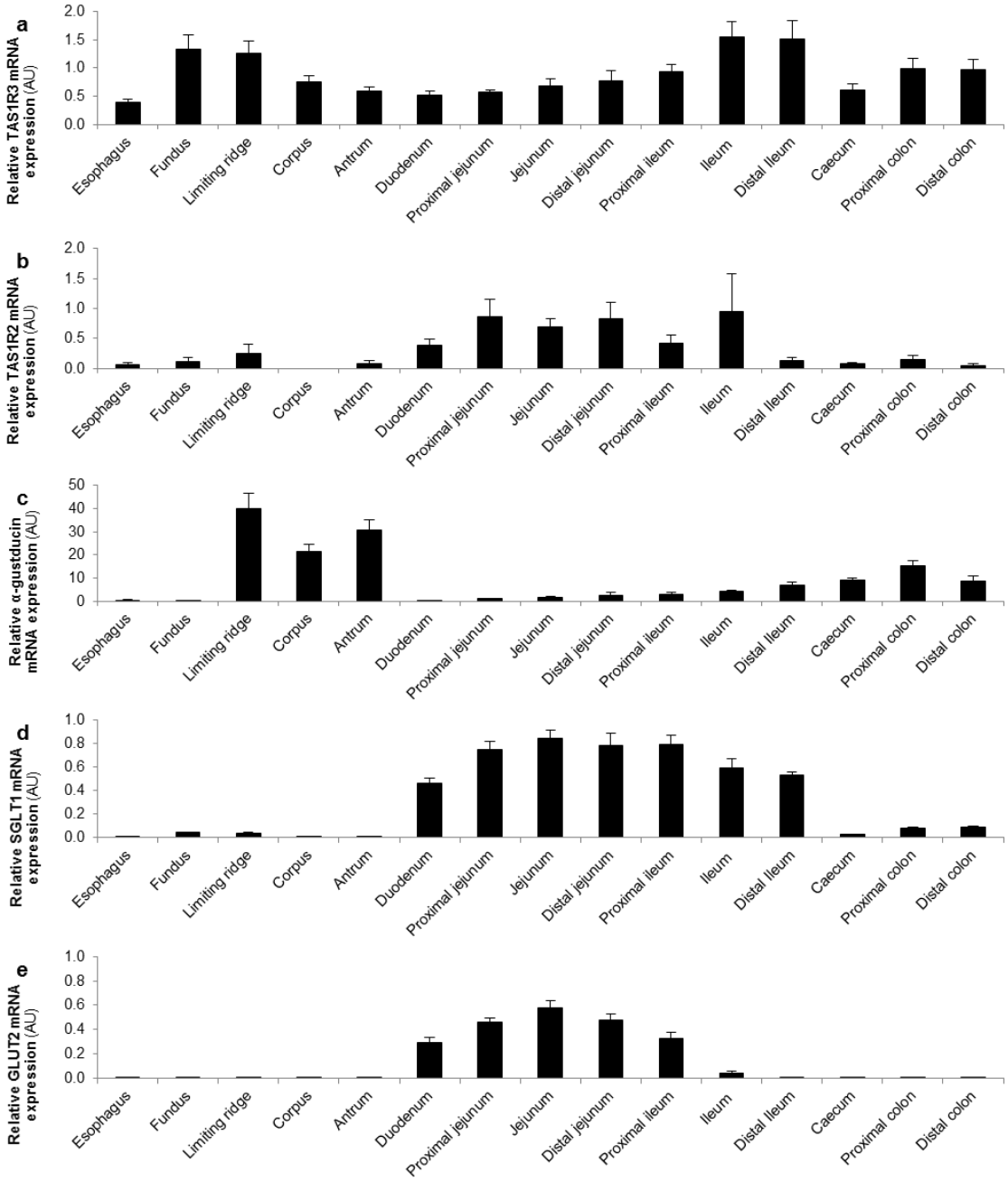


Figure 3. TAS1R3 and α-gustducin are expressed throughout the GI tract while TAS1R2 and the glucose transporters are expressed in the small intestine. Relative mRNA expression levels of (a, b) the two subunits of the sweet taste receptor (TAS1R2-TAS1R3), (c) α-gustducin and (d, e) the glucose (SGLT1, GLUT2) transporters throughout the mouse GI tract (n=5). Results are presented as mean ± SEM. AU: arbitrary units.

The differential expression of the TAS1R3 subunit (corpus and jejunum) and the TAS1R2 subunit (only jejunum) allowed us to investigate, in the respective *ex vivo* segments, whether the effect of glucose and the high- and low-intensity sweeteners on ghrelin release is region-dependent and thus involves the TAS1R2-TAS1R3 receptor heterodimer. Furthermore, the effect of the compounds was tested in segments from WT and α-gust$^{-/-}$ mice to elicit the role of the G-protein, α-gustducin, coupled to the sweet taste receptor complex.

3.5.2.1 D-glucose decreased ghrelin release from gastric and jejunal segments in an α-gustducin-independent manner

D-glucose (200mM) tended to or decreased octanoyl ghrelin release (WT: P=0.076; α-gust$^{-/-}$: P<0.01) from segments of the corpus of WT and α-gust$^{-/-}$ mice (Figure 4a). A similar effect was observed in jejunal segments from both WT and α-gust$^{-/-}$ mice (Figure 4b). Accordingly D-glucose decreased total ghrelin release from segments of the corpus (WT; P<0.01, α-gust$^{-/-}$; P<0.001) and jejunum (WT; P<0.05, α-gust$^{-/-}$; P<0.05) (Figure 4c, d). No interaction effects (genotype*treatment) were observed.

3.5.2.2 Oligofructose decreased ghrelin release from gastric and jejunal segments in an α-gustducin-independent manner

OFS (10%) tended to or decreased (WT: P=0.076; α-gust$^{-/-}$: P<0.05) octanoyl ghrelin release from segments of the corpus in both genotypes (Figure 4e). A significant (P<0.05) OFS-induced reduction in octanoyl ghrelin release was also observed in jejunal segments (Figure 4f). Accordingly, OFS significantly decreased total ghrelin release from segments of the corpus and jejunum from both genotypes (Figure 4g, h). No interaction effects (genotype*treatment) were observed.

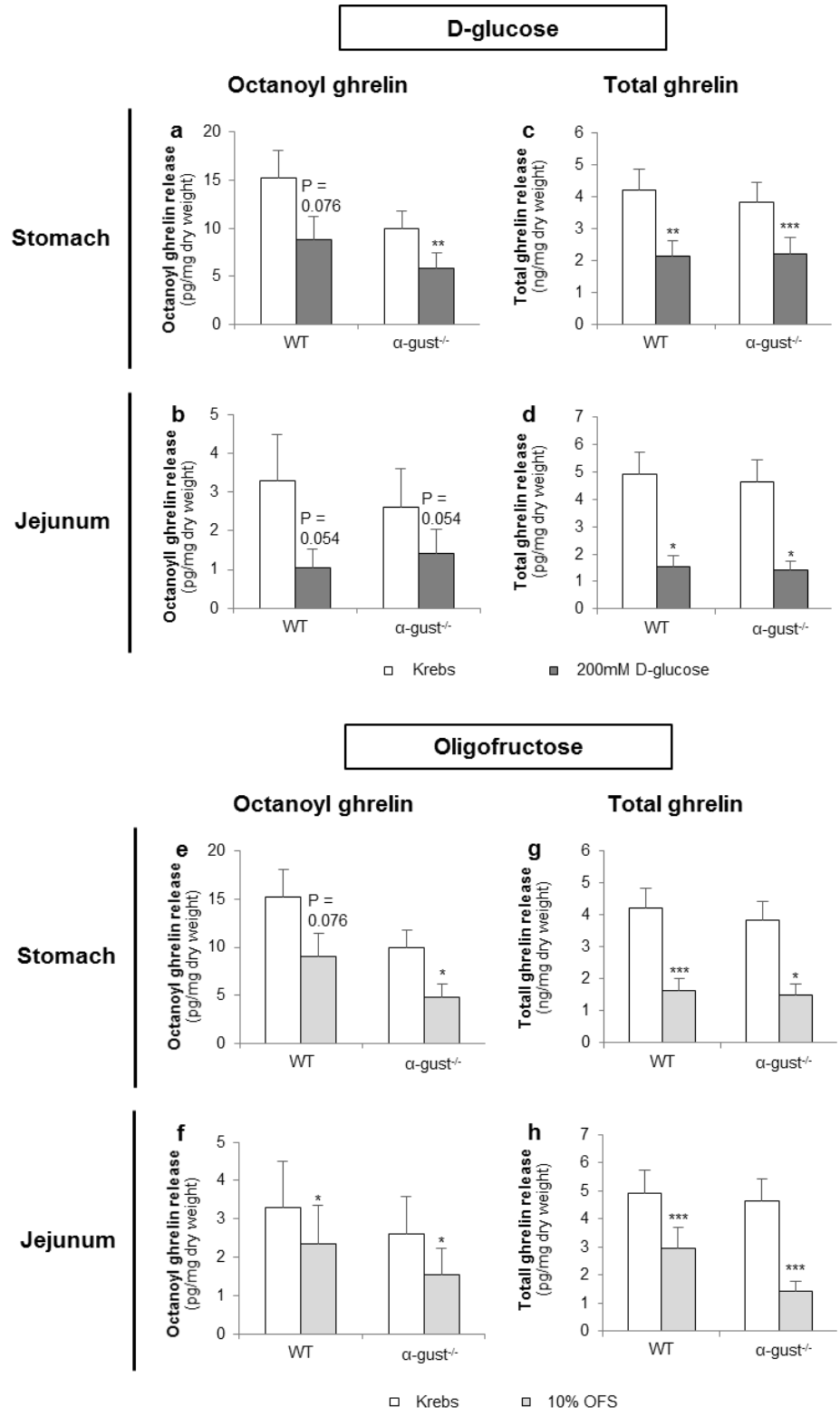


Figure 4. The effect of D-glucose and oligofructose on ghrelin release from segments of the corpus and jejunum is α-gustducin-independent. Effect of a 2h stimulation with Krebs buffer or 200mM D-glucose (upper panel) or 10% oligofructose (lower panel) on octanoyl (left panels) and total ghrelin release (right panels) from tissue segments of the corpus of the stomach (n=6) or the jejunum (n=6) from WT and α-gust$^{-/-}$ mice. Results are presented as mean ± SEM. * P<0.05, ** P<0.01, *** P<0.001 vs. Krebs treated segments

3.5.2.3 Sucralose increased octanoyl ghrelin, but not total ghrelin release from gastric and jejunal segments in an α-gustducin-independent manner

Sucralose (200mM) significantly increased octanoyl ghrelin release from segments of the corpus and jejunum of both genotypes (Figure 5a, b). However, sucralose did not affect total ghrelin release from segments of either the corpus or jejunum in both genotypes (Figure 5c, d). No interaction effects were observed.

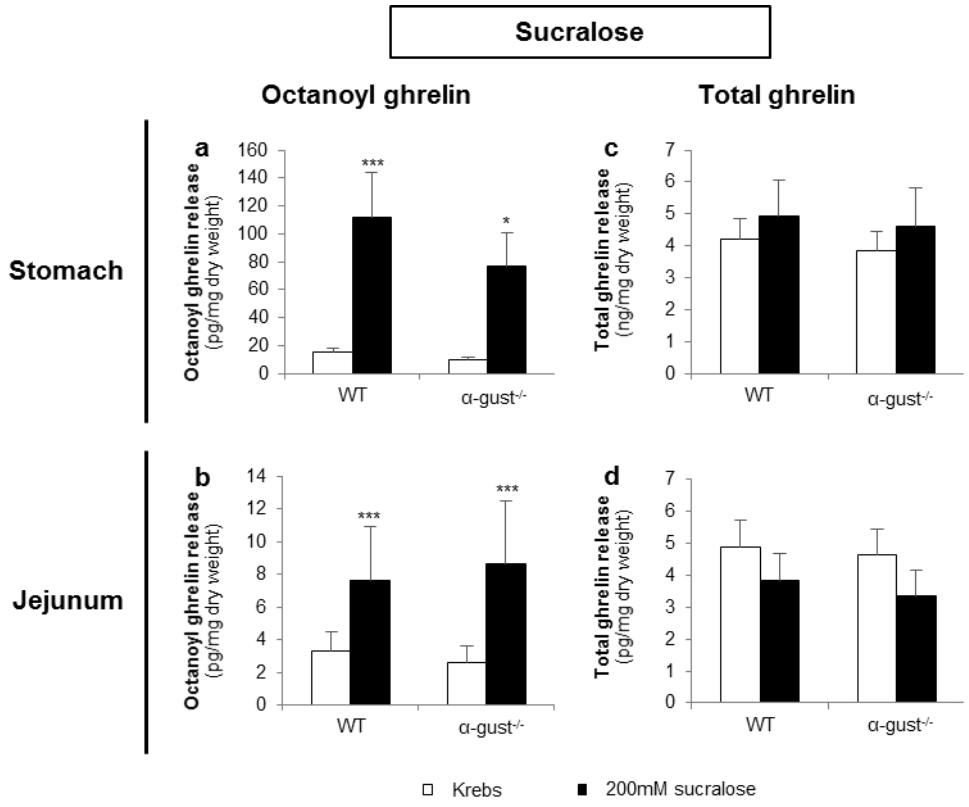


Figure 5. Sucralose increased octanoyl ghrelin, but not total ghrelin release from gastric and jejunal segments in an α-gustducin-independent manner. Effect of a 2h stimulation with 200mM sucralose or Krebs buffer on (a, b) octanoyl and (c, d) total ghrelin release from tissue segments of the corpus of the stomach (n=6) or the jejunum (n=6) from WT and α-gust$^{-/-}$ mice. Results are presented as mean ± SEM. * P<0.05, *** P<0.001 vs. Krebs treated segments.

3.5.3 In vivo studies in mice

The effect of the caloric and non-caloric sweeteners was tested *in vivo* in WT and α-gust$^{-/-}$ mice to investigate the physiological role of sweet taste receptor activation.

3.5.3.1 The sensing of D-glucose by the ghrelin cell is polarized and occurs via the lumen

Basal fasted octanoyl ghrelin levels were 41% lower (P<0.05) in α-gust$^{-/-}$ control mice, compared to WT control mice. Intragastric administration of D-glucose (4g/kg) in fasted mice significantly decreased plasma octanoyl (WT: -41±11%; α-gust$^{-/-}$: -48±5%) and total ghrelin levels (WT: -38±8%; α-gust$^{-/-}$ -48±4%) in both genotypes compared to vehicle-treated mice (Figure 6a, b). This was accompanied by an increase in duodenal tissue octanoyl ghrelin content in both WT (P<0.01) and α-gust$^{-/-}$ mice (P<0.05) but not in gastric tissue octanoyl ghrelin content (Figure 6c, d).

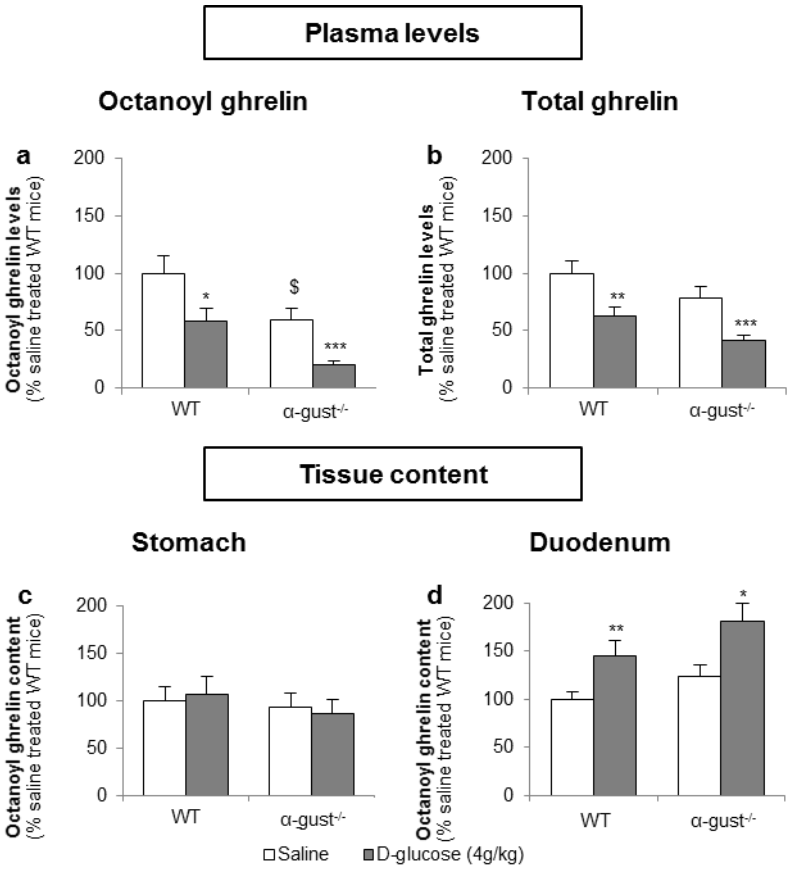


Figure 6. The inhibition of plasma ghrelin levels after an intragastric administration of D-glucose originates mainly from duodenal ghrelin cells. WT and α-gust$^{-/-}$ mice were gavaged with D-glucose (4g/kg) (n=8) or saline (n=13). Ghrelin levels were determined in (a, b) plasma, (c) stomach and (d) duodenum, 40 min after administration. Results (mean ± SEM) are expressed relative to the control stimulation (saline treated WT mice). * P<0.05, ** P<0.01 and *** P<0.001 vs. saline, $ P<0.05 vs. saline treated WT mice.

To determine whether D-glucose is sensed via the lumen or the bloodstream after glucose uptake, the effect of intragastric (4g/kg) versus intravenous administration of D-glucose (1g/kg) on ghrelin release was compared. A pilot experiment showed that 1g/kg D-glucose induced comparable peak blood glucose levels (330±15 mg/dl) compared to the intragastric administration of 4g/kg D-glucose (317±31 mg/dl). Intravenous administration of D-glucose neither affected plasma octanoyl or total ghrelin levels nor gastric or duodenal tissue octanoyl ghrelin content (Figure 7a-d).

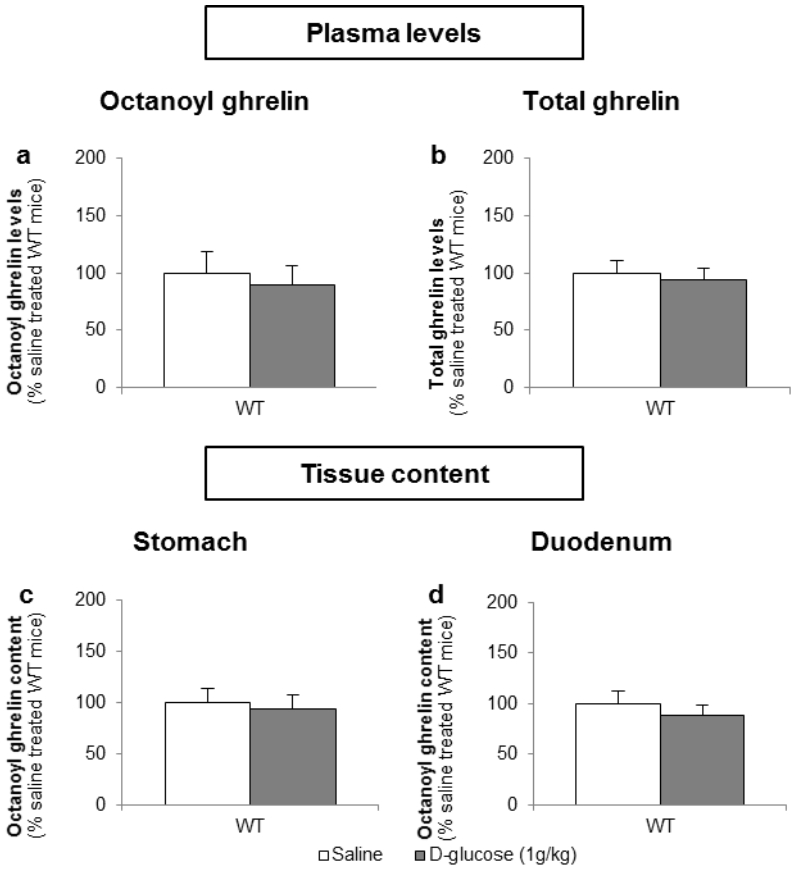


Figure 7. The sensing of D-glucose is polarized and occurs at the luminal side. Mice were intravenously injected with D-glucose (1g/kg) (n=10) or saline (n=11). Ghrelin levels were determined in (**a, b**) plasma, and in protein extracts from the (**c**) stomach and (**d**) duodenum, 40 min after administration. Results (mean ± SEM) are expressed relative to the control stimulation (saline treated WT mice).

3.5.3.2 Intragastric administration of neither a low- nor a high-potency sweetener affected plasma ghrelin levels

In contrast to D-glucose, intragastric administration of oligofructose (OFS) (5.6g/kg) or sucralose (9.0mg/kg) did not affect plasma octanoyl or total ghrelin levels in either genotype (Figure 8a-d).

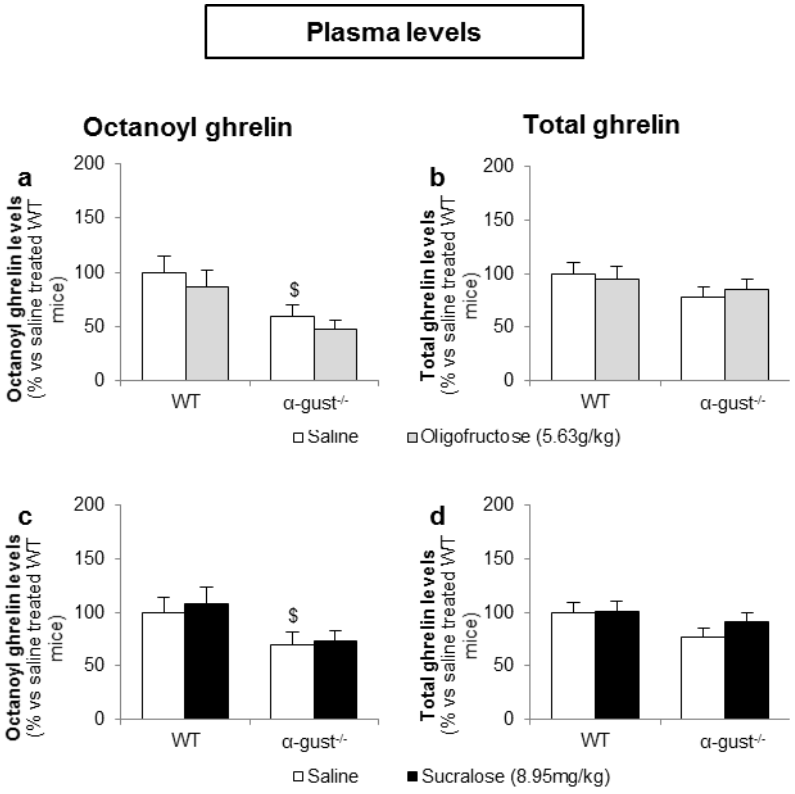


Figure 8. Intragastric administration of sweeteners does not affect ghrelin release. WT and α-gust$^{-/-}$ mice were gavaged (**a, b**) oligofructose (5.63 g/kg) (n=8), (**c, d**) sucralose (8.95mg/kg) (n=9) or saline (n=9-13). Plasma octanoyl and total ghrelin levels were determined 40 min after administration. Results (mean ± SEM) are expressed relative to the control stimulation (saline treated WT mice). $ P<0.05 vs. vehicle treated WT mice.

3.6 Discussion

The *in vitro* results in the ghrelinoma cell line and *ex vivo* results obtained in tissue segments showed that D-glucose and oligofructose (OFS) reduced ghrelin release at concentrations physiological to the postprandial luminal fluid. In contrast, the artificial sweetener sucralose increased ghrelin release at the supraphysiological concentration of 200mM. Furthermore, neither α-gustducin mediated sweet taste receptor signaling nor glucose transport (SGLT1, GLUT family) played a role in the effect of D-glucose, OFS or sucralose on ghrelin release.

Our *in vivo* findings indicate that the sensing of D-glucose by the X/A cells is polarized and occurred at the luminal side. The glucose-induced reduction in plasma ghrelin levels is α-gustducin-independent and originates from a reduced ghrelin release from duodenal, but not gastric cells. In contrast, the low-and high-intensity sweeteners (OFS and sucralose) did not elicit any changes in plasma ghrelin levels.

Previous studies showed that concentrations of D-glucose physiological to the postprandial basolateral concentrations were able to elicit changes in ghrelin secretion.

For instance, Sakata *et al.* showed that, compared to a low glycemic state (1mM), normoglycemic (5mM) and high (10mM) concentrations of D-glucose decreased octanoyl ghrelin secretion from primary cultures of gastric mucosal cells, respectively (Sakata et al., 2012). Oya *et al.* showed that low (1mM), normoglycemic (5mM) and high (10mM) concentrations of D-glucose increased ghrelin secretion compared to 25mM D-glucose in MGN3-1 cells (Oya et al., 2015).
We could not confirm these findings and only observed an inhibition of ghrelin secretion at 200mM D-glucose both *in vitro* and *ex vivo*. This dose mimics luminal glucose concentrations which range between 50-500mM (Ferraris et al., 1990). A similar observation was made for oligofructose which is usually supplemented at a dose of 8-21g/day in the diet or in a drink, and results in 5-10% OFS in the luminal fluid (Liber and Szajewska, 2013). These findings suggest that the sensing of D-glucose and OFS may occur at the luminal side of the intestinal epithelium. This was confirmed by our *in vivo* studies which showed that intravenous administration of D-glucose did not affect plasma ghrelin levels. Immunohistochemistry studies previously showed that in contrast to “closed-type “ghrelin cells, which are not in contact with the lumen, “open-type” ghrelin cells show the presence of the TAS1R3-subunit in their apical cell pole contacting the lumen (Hass et al., 2010). Since sweet taste receptors on the tongue are typically activated by 30–1000mM glucose (Damak et al., 2003, Reimann et al., 2008), the apical localization of the TAS1R3 subunit could explain the luminal sensing of D-glucose. Nevertheless, many open-type duodenal ghrelin cells also showed TAS1R3 staining in their basolateral domain. We have previously shown that in contrast to glucose, the sensing of amino acids is not polarized (Vancleef et al., 2015). Since TAS1R3 is also involved in amino acid sensing, it is likely that the TAS1R3 staining in the basolateral domain is selectively involved in amino acid sensing.

The amount of sucralose in sweetened soft drinks represents about 0.4mM and sucralose typically activates the sweet taste receptor at low millimolar concentrations (Damak et al., 2003). Our findings therefore indicate that sucralose only stimulated ghrelin release at supraphysiological concentrations (200mM).

The effect of sucralose on ghrelin release in the ghrelinoma cell line and *ex vivo* segments was opposite to those of D-glucose and OFS. Functional studies of the sweet taste receptor have revealed at least four binding sites for sweet-tasting compounds (Sanematsu et al., 2014). It is likely that low-

intensity sweeteners (glucose and OFS) and high-intensity sweeteners such as sucralose will bind to a different binding site, possibly activating a different signaling cascade. Sugars are thought to increase cAMP levels while artificial sweeteners may act by increasing levels of IP3 (Bernhardt et al., 1996). Still, both the cAMP and IP3 cascades eventually result in increased Ca^{2+} levels in the cell (Bernhardt et al., 1996) and cannot explain why glucose and OFS decrease ghrelin release and sucralose stimulates ghrelin release.

Sucralose (> 0.62mM) has a bitter taste quality in rats (Torregrossa et al., 2015), but not in humans (Schiffman et al., 1995). However, it cannot be excluded that at high concentrations (200mM) surcralose might also taste bitter in mice and humans. Activation of bitter taste receptors has been shown to stimulate ghrelin secretion *in vivo* in mice, partially via α-gustducin (Janssen et al., 2011). Since sucralose stimulated ghrelin secretion in segments from both WT and α-gust$^{-/-}$ mice, it unlikely that the effect of sucralose is mediated via bitter taste receptors. Sucralose specifically increased octanoyl, but not total and thus desoctanoyl ghrelin release, therefore it might exert its activity through modulation of the activity of ghrelin-O-acyl transferase (GOAT). No evidence in literature so far supports the hypothesis of a link between sucralose and GOAT activity.

We could not assign an important role of the TAS1R2-TAS1R3 heterodimer in the effect of glucose and sweeteners on gastric ghrelin release. Indeed, the mRNA expression of the TAS1R2 subunit was absent in segments from the corpus and in the ghrelinoma cell line, which is of gastric origin. Other studies using the TAS1R2-lacZ knock-in mouse did not observe TAS1R2 expression in the stomach (Iwatsuki et al., 2010). In contrast, Koyama *et al*. showed a very low expression of TAS1R2 in the MGN3-1 cell line and primary gastric ghrelin cells using RNA sequencing (Koyama et al., 2016). However, TAS1R3 may also function as a homodimer, as previously shown on the tongue (Zhao et al., 2003), in adipocytes (Masubuchi et al., 2013) and in pancreatic β-cells (Nakagawa et al., 2014). Zhao *et al*. showed that the TAS1R3 homodimer was not able to detect sweeteners and carbohydrates at low concentrations (<300mM) (Zhao et al., 2003). This may explain why only high concentrations of glucose (200mM), sucralose (200mM) and oligofructose (10%) affected ghrelin secretion in the MGN3-1 cells.

However, the lack of effect of the sweet taste receptor antagonist, gurmarin, which has been shown to block the TAS1R3 subunit (Vancleef et al., 2015, Daly et al., 2013), on glucose and sweetener induced ghrelin release suggests that neither TAS1R2-TAS1R3 nor the TAS1R3 homodimer is important. Furthermore, the effect of D-glucose and the sweeteners did not differ between segments from WT and α-gust$^{-/-}$ mice, indicating that gustducin mediated signaling does not play an important role. However, it cannot be excluded that the sweet taste receptor heterodimer or the TAS1R3 homodimer can couple to other G-proteins than α-gustducin. Indeed, α-gust$^{-/-}$ mice are not completely unresponsive to sweet compounds (Danilova et al., 2006) and the TAS1R3 homodimer has been shown to couple to Gs in adipocytes (Masubuchi et al., 2013). Furthermore, indirect effects,

mediated via glucose-induced GLP-1 release, seem unlikely since the effect should be blunted in segments from α-gust$^{-/-}$ mice (Jang et al., 2007).

In L-cells, both α–gustducin mediated sweet taste receptor signaling and the glucose transporter, SGLT1, mediate glucose-induced GLP-1 secretion (Jang et al., 2007, Gorboulev et al., 2011). However, inhibitors for SGLT1 and the GLUT family could not confirm an involvement of these proposed glucose-sensors in the effect of D-glucose on octanoyl ghrelin secretion. Previous studies also suggested that K_{ATP} channels are involved in the effect of 25mM D-glucose on ghrelin secretion in MGN3-1 cells (Oya et al., 2015). However, tolbutamide (a potassium channel blocker) and diazoxide (a potassium channel activator) neither enhanced nor inhibited 1, 5 or 10mM glucose-induced ghrelin secretion in primary cultures of gastric mucosal cells (Sakata et al., 2012).

Our *in vivo* studies showed that the glucose-induced ghrelin inhibition was due to a tissue-specific inhibition of octanoyl ghrelin release from the duodenum. Williams *et al*. showed that intragastric infusion of glucose or water inhibited ghrelin release when gastric emptying was permitted but not when emptying was prevented, indicating that gastric chemosensation is not a sufficient trigger for the ghrelin response (Williams et al., 2003). Thus although our *in vitro* and *ex vivo* studies indicate that glucose can inhibit ghrelin secretion in the stomach, *in vivo* this glucose sensing may be ineffective.

Parker *et al*. showed that an intraduodenal glucose infusion proved to be just as effective in suppressing ghrelin levels as an intragastric infusion in healthy older men and women (Parker et al., 2005). The magnitude of the glucose-induced decrease in plasma ghrelin levels was even dependent on the length of the small intestine exposed (Little et al., 2006). Tamboli *et al.* showed that jejunal glucose administration suppressed ghrelin levels to a greater degree compared with an intagrastric glucose administration in obese subjects. This was independent of circulation glucose levels, indicating that a nutrient-initiated signal in the jejunum may have regulated ghrelin secretion in this study (Tamboli et al., 2016). These results indicate that although the primary source of ghrelin is the gastric mucosa, small intestinal nutrient exposure is sufficient to decrease postprandial ghrelin levels.

The sweeteners OFS and sucralose were not able to affect plasma ghrelin levels or gastric or duodenal ghrelin content *in vivo*. These findings, together with the observation that the effect of D-glucose on ghrelin levels is not dependent on signaling through α-gustducin, would argue against a role for α-gustducin mediated sweet taste receptor signaling as glucose sensor of the X/A cell. Also in a dose-escalation study from 0 to 55 g daily of OFS, no significant effects were observed on plasma ghrelin levels (Pedersen et al., 2013). Artificial sweeteners did also not elicit differences in plasma ghrelin levels in healthy subjects in previous studies (Steinert et al., 2011a, Brown et al., 2011). Furthermore, results comparing equicaloric doses of glucose and fructose observed that the decrease in ghrelin levels after fructose administration, which is sweeter than glucose, was less pronounced

(Prodam et al., 2006) or equal (Bowen et al., 2006) to the effect of glucose. These results suggest that the effect of glucose and fructose is not determined by their sweetness.
In fact, a similar discrepancy has been found for the effect of sucralose on GLP-1 release in *in vitro* and *in vivo* studies. In enteroendocrine cell lines sucralose stimulates GLP-1 release via the sweet taste receptor (Jang et al., 2007, Ohtsu et al., 2014) whereas *in vivo* studies in humans and rodents fail to demonstrate an effect of sucralose on GLP-1 release (Steinert et al., 2011a, Wu et al., 2012). The regulatory interface of the GI tract is more complex than the physiological processes mimicked in *in vitro* experiments and is modulated by multiple homeostatic and non-homeostatic factors. This complexity may explain the discrepancy between *in vitro* and *in vivo* findings.

3.7 Conclusions

In conclusion, sensing of D-glucose by the ghrelin cell is polarized, occurs at the luminal side of the duodenum and may overrule gastric glucose sensing. Furthermore, α-gustducin-mediated sweet taste receptor signaling does not play a physiological role in the sensing of carbohydrates and sweeteners by the ghrelin cell since; 1) the effects of D-glucose and sweeteners in the ghrelinoma cell line are not blocked by the sweet taste receptor antagonist gurmarin, 2) D-glucose and the sweeteners affect ghrelin release in gastric segments which do not express one of the subytpes (TAS1R2) of the sweet taste receptor, 3) the effects are not reduced in α-gust$^{-/-}$ mice and 4) the sweeteners oligofructose and sucralose were not able to elicit the same responses on ghrelin secretion as D-glucose *in vivo*.

We were unable to show a role for SGLT1 or GLUT2 as glucose sensor of the ghrelin cell and prior data on the involvement of the K_{ATP} channel are inconclusive. Therefore, the role of different G-proteins and the functional role of a TAS1R3 homodimer or K_{ATP} channels as glucose sensors of the ghrelin cell warrant further investigation.

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Author Contributions: “S.S. and I.D. conceived and designed the experiments; S.S. and L.V. performed the experiments; S.S. analyzed the data; I.D. contributed reagents/materials/analysis tools; S.S. and I.D. wrote the paper.”

Conflicts of Interest: The authors declare no conflict of interest.

Chapter 4

EXPLORING THE ROLE OF GUSTDUCIN-MEDIATED GUT HORMONE RELEASE IN THE EFFECT OF SWEETENERS (OLIGOFRUCTOSE AND SUCRALOSE) ON HIGH-FAT DIET INDUCED BODY WEIGHT GAIN

The data represented in the following chapter are published in Molecular Nutrition & Food Research:

Supplementation of oligofructose, but not sucralose, decreases high-fat diet induced body weight gain in mice independent of gustducin-mediated gut hormone release

Steensels S., Cools L., Avau B., Vancleef L., Farré R., Verbeke K., Depoortere I.

Mol Nutr Food Res. **2016**; doi: 10.1002/mnfr.201600716.

4 EXPLORING THE ROLE OF GUSTDUCIN-MEDIATED GUT HORMONE RELEASE IN THE EFFECT OF SWEETENERS (OLIGOFRUCTOSE AND SUCRALOSE) ON HIGH-FAT DIET INDUCED BODY WEIGHT GAIN

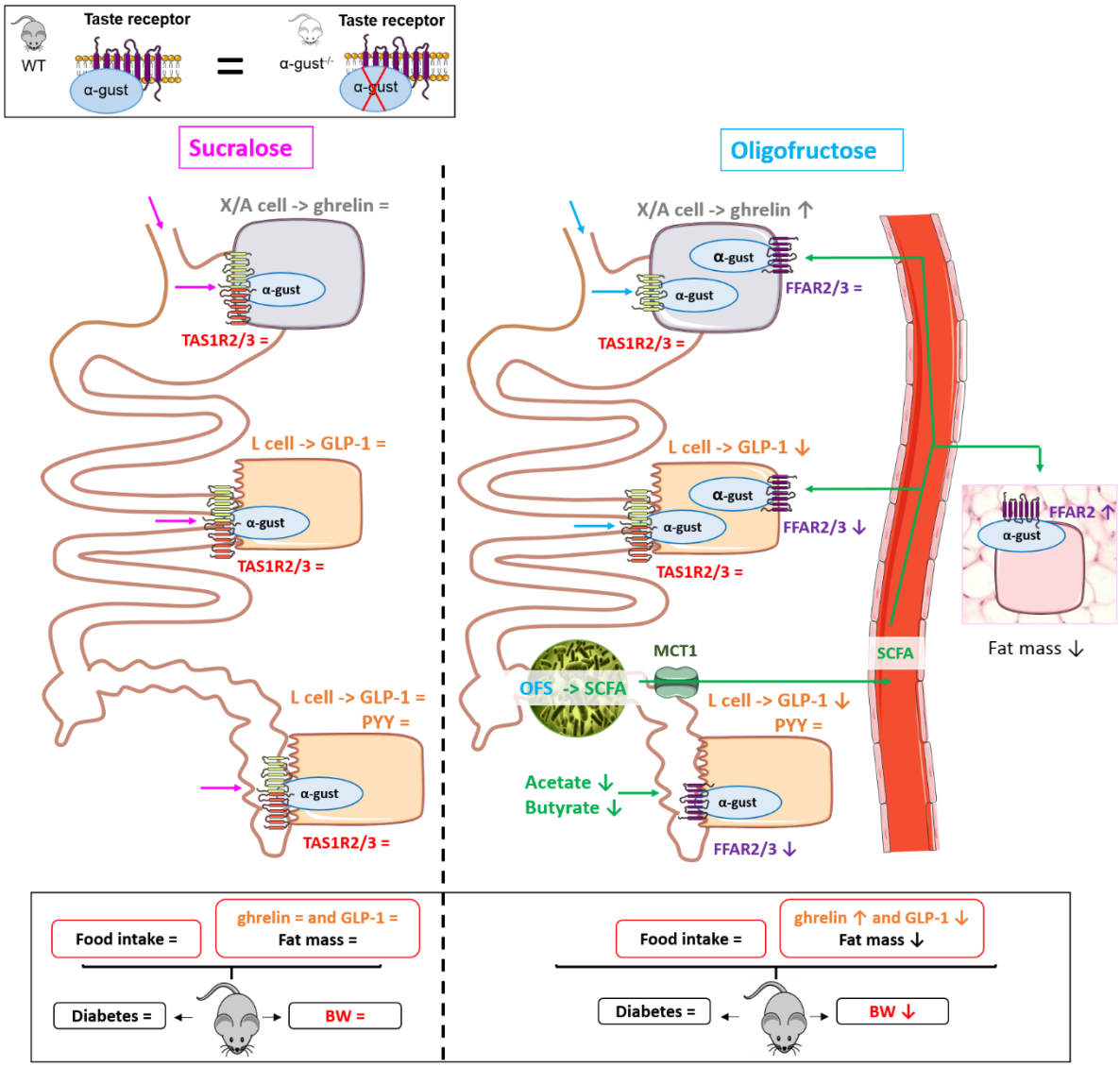
4.1 Abstract

Scope: Enteroendocrine cells (EECs) sense nutrients through taste receptors similar to those on the tongue. Sweet -and fatty acid taste (FFAR) receptors coupled to the gustatory G-protein, gustducin, on EECs play a role in gut hormone release. We studied if supplementation of artificial (sucralose) or prebiotic (oligofructose; OFS) sweeteners target gustducin-mediated signaling pathways to alter gut hormone release and reduce obesity-associated disorders.

Methods and results: Wild type (WT) and α-gustducin knockout (α-gust$^{-/-}$) mice were fed a high-fat diet and gavaged once daily (8 weeks) with water or equisweet concentrations of sweeteners. OFS but not sucralose decreased body weight gain (-19±3%, $P<0.01$), fat pad mass (-55±6%, $P<0.001$) and insulin resistance (-39±5%, $P<0.001$) independent of α-gustducin. Neither sweetener improved glucose intolerance, while solely OFS improved the disturbed colonic permeability. OFS decreased (-65±8%, $P<0.001$) plasma GLP-1 but not ghrelin and PYY levels in WT mice. Caecal acetate and butyrate levels were reduced by OFS in both genotypes suggesting enhanced uptake of short-chain fatty acids which may target FFAR2 (upregulated expression) in adipose tissue.

Conclusion: OFS, but not sucralose, reduced body weight gain and decreased intestinal permeability, but not glucose intolerance. Effects were not mediated by altered gut hormone levels or gustducin-mediated signaling.

4.2 Graphical abstract



Artificial sweeteners do not affect gut hormone levels and are metabolically inert in mice on a high fat diet. In contrast, prebiotic oligosaccharides (OFS) prevent body weight gain but not glucose intolerance. Alterations in sweet and short-chain fatty acid receptors (FFAR) (studied in WT and α-gust$^{-/-}$ mice) which regulate gut hormone levels are not mandatory for the positive effects of OFS. Enhanced uptake of SCFAs may favor interaction with FFAR2/3 on adipose tissue to induce weight loss.

4.3 Introduction

Obesity is posing a major healthcare problem to our modern society. Excessive sugar consumption is a major contributor to the obesity epidemic. Therefore guidelines from the WHO recommend a reduction in sugar intake (Organization, 2015). Sugar replacers, such as the artificial sweetener sucralose, can help reduce the sugar content of meals without affecting its taste. Artificial sweeteners are non-caloric but may not be metabolically inert. Epidemiological studies found a positive correlation (Fowler, 2016) or no correlation (Duran Aguero et al., 2014) between sweetener intake and weight gain. Two large randomized controlled trials showed that replacing sugar-containing beverages with noncaloric sweetened beverages reduced weight gain in children and adults (de Ruyter et al., 2012, Tate et al., 2012). Next to these artificial sweeteners, prebiotic sweeteners such as oligofructose (OFS), have been proposed as functional food ingredients that modulate the secretion of gut hormones to prevent obesity and obesity-related diseases in rodents (Cani et al., 2004a, Cluny et al., 2015, Cani et al., 2009). In humans, the link between OFS and body weight loss is less straightforward with studies reporting no effect (Pedersen et al., 2013, Liber and Szajewska, 2014) or a modest positive effect (Parnell and Reimer, 2009) on body weight loss after OFS supplementation.

EECs in the gut sense nutrients through taste receptors (TASR) and chemosensory signaling pathways similar to those present on the tongue (Depoortere, 2014, Janssen and Depoortere, 2013). Both OFS and sucralose can activate the sweet TASR (consisting of the TAS1R2-TAS1R3 heterodimer) which is present on X/A cells secreting the hunger hormone ghrelin and L-cells secreting the satiety hormones, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), and the trophic hormone glucagon-like peptide-2 (GLP-2) to affect gut hormone release (Hass et al., 2010, Jang et al., 2007).

In addition, colonic fermentation of OFS by the gut microbiota will increase the production of short-chain fatty acids (SCFAs). SCFAs may induce satiety by stimulating the release of GLP-1 and PYY (Canfora et al., 2015) via activation of the SCFA receptors FFAR2 and FFAR3 on the L-cells. Indeed, *in vitro* and *in vivo* studies using FFAR2$^{-/-}$ and FFAR3$^{-/-}$ mice showed reduced SCFA-induced GLP-1 secretion together with a parallel impairment of glucose tolerance (Tolhurst et al., 2012). Furthermore, circulating SCFAs mainly inhibit the secretion of the orexigenic hormone ghrelin through FFAR2 receptor activation (Engelstoft et al., 2013). α-*gust*ducin, the α-subunit of the G-protein coupled to TASRs, plays an important role in taste transduction (McLaughlin et al., 1992) and is colocalized with TAS1R2-TAS1R3, FFAR2 and FFAR3 on L-cells (Jang et al., 2007, Li et al., 2013). Accordingly, glucose and SCFA-induced GLP-1 release is blunted in α-*gust*ducin knockout (α-*gust*$^{-/-}$) mice (Jang et al., 2007, Li et al., 2013).

In addition to the increased incretin production, oligofructose may improve inflammation and metabolic disorders associated with obesity by lowering intestinal permeability through a mechanism involving GLP-2 release from intestinal L-cells (Cani et al., 2009). Since GLP-2 is co-secreted with GLP-1, we hypothesized that the GLP-2 induced improvement in intestinal permeability would be decreased in α-gustducin knockout mice. Furthermore, the trophic effects of GLP-2 and the SCFAs produced during fermentation of oligofructose may increase mucosal thickness (Kripke et al., 1989). This may affect both the number of EECs and hence plasma levels of gut hormones, and the intestinal barrier function by increasing paracellular transport.

This study aimed to investigate whether daily intragastric administration of artificial (sucralose) or natural (OFS) sweeteners can prevent high-fat diet induced body weight gain, glucose intolerance and impairment of gut permeability by interacting with extra-oral taste receptors specifically coupled to α-gustducin. In addition, we performed an in-depth study on the origin of the altered gut hormone levels which may involve changes in enteroendocrine plasticity induced by an increased mucosal thickness. By using equisweet concentrations we aimed to get the same degree of sweet taste receptor activation allowing us to discriminate between the role of sweet taste receptor-induced (activated by both sweeteners) and short chain fatty acid taste receptor-induced (only activated by the fermentation products of OFS) effects.

4.4 Materials and Methods

4.4.1 Animals

Male C57BL/6 wild type (WT) mice and α-gust$^{-/-}$mice (Dr. R. Margolskee, Monell Chemical Senses Center, Philadelphia, United States) were kept in the animal facility (20–22 °C) under a 14-h:10-h light-dark cycle and had ad libitum access to food and water. All experimental procedures were approved by the Ethical committee for Animal Experiments of the KU Leuven (P100-2013).

4.4.2 Experimental design

6 week-old WT and α-gust$^{-/-}$mice were fed a high-fat diet (HFD; 60% kcal fat, D12492, Research Diets) and gavaged once daily (1 hour before the dark phase) with water or equisweet solutions of 300mg OFS (oligofructose, Beneo orafti) or 0.52mg sucralose (Sigma-Aldrich) for 8 weeks (n=9/group). A daily dose of 300mg OFS was used, since previous studies showed metabolic improvements in rodents fed a HFD supplemented with 10% OFS which corresponds to a daily intake of ±300mg OFS (Everard et al., 2011).

4.4.3 In vivo analyses

Body weight and food intake were monitored during 8 weeks. An oral glucose tolerance test (OGTT) was performed after 6 weeks of treatment and blood glucose and serum insulin levels were measured. After 8 weeks, fasted (6h) mice were gavaged with 2g D-glucose/kg, 10 min before sacrifice.

4.4.4 Biochemical analyses

Blood was taken by cardiac puncture and supplemented with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (4mM) and ethylenediaminetetraacetic acid (1mg/ml). Plasma was supplemented with dipeptidyl peptidase-4 inhibitor (10µl/ml) (for GLP-1 and PYY) or acidified (10% with 1N hydrochloride) (for ghrelin). The stomach, small intestine, colon and fat deposits were collected and divided in predefined parts which were used for immunohistochemistry (fixed in 4% paraformaldehyde), or RT-PCR (overnight incubation in RNALater (Qiagen)) and stored at -80°C for further analysis. Plasma ghrelin, GLP-1, PYY and leptin levels were measured by radioimmunoassay (RIA) and ELISA respectively. Caecal content was used for SCFA analyses, and a part of the distal colon was immediately used for permeability experiments. See expanded Materials and Methods for a detailed description.

4.4.5 Statistical analyses

All values are represented as mean±SEM. Data was assessed for normality and homogeneity of distribution using histograms and Q-Q plots; as the data were non-normally distributed, log-transformation was applied. Changes in body weight, food intake, glucose tolerance and plasma insulin levels were analyzed on log transformed data using a repeated measures mixed models analysis (SAS Studio University Edition). TEER and fluorescein levels over time between different genotypes and treatments were analyzed using a repeated measures mixed models analysis. Other data were analyzed with a two-way ANOVA, followed by planned comparisons post-hoc testing and Bonferroni correction (Statistica 12, Statsoft). Prism (GraphPad Software) was used to calculate the area under the curve (AUC) for the OGTT and plasma insulin levels over time. Significance was accepted at the 5% level.

4.5 Results

4.5.1 OFS, but not sucralose prevents body weight gain from a HFD

Daily gavage of OFS, but not sucralose, for 8 weeks decreased body weight gain in WT (-19±3%, treatment*time $P<0.01$) mice and tended to decrease body weight gain in α-gust$^{-/-}$ mice (-14±2%, treatment*time $P=0.055$) (Figure 1A, B). The effect was significant from week 4

on in WT mice (P<0.05), and at week 8 in α-gust$^{-/-}$mice (P<0.01). This correlates with plasma leptin levels which were decreased from week 6 on in WT (P<0.01) but not in α-gust$^{-/-}$mice (treatment*genotype P<0.05) (Figure 1E, F).

OFS but not sucralose administration reduced (P<0.001) total fat pad mass (WT:-55±6%; α-gust$^{-/-}$;-41±7%) (Figure 1C, D) and plasma leptin levels (P<0.001) at sacrifice (8 weeks), in both genotypes to a similar extent (Figure 1F, G).

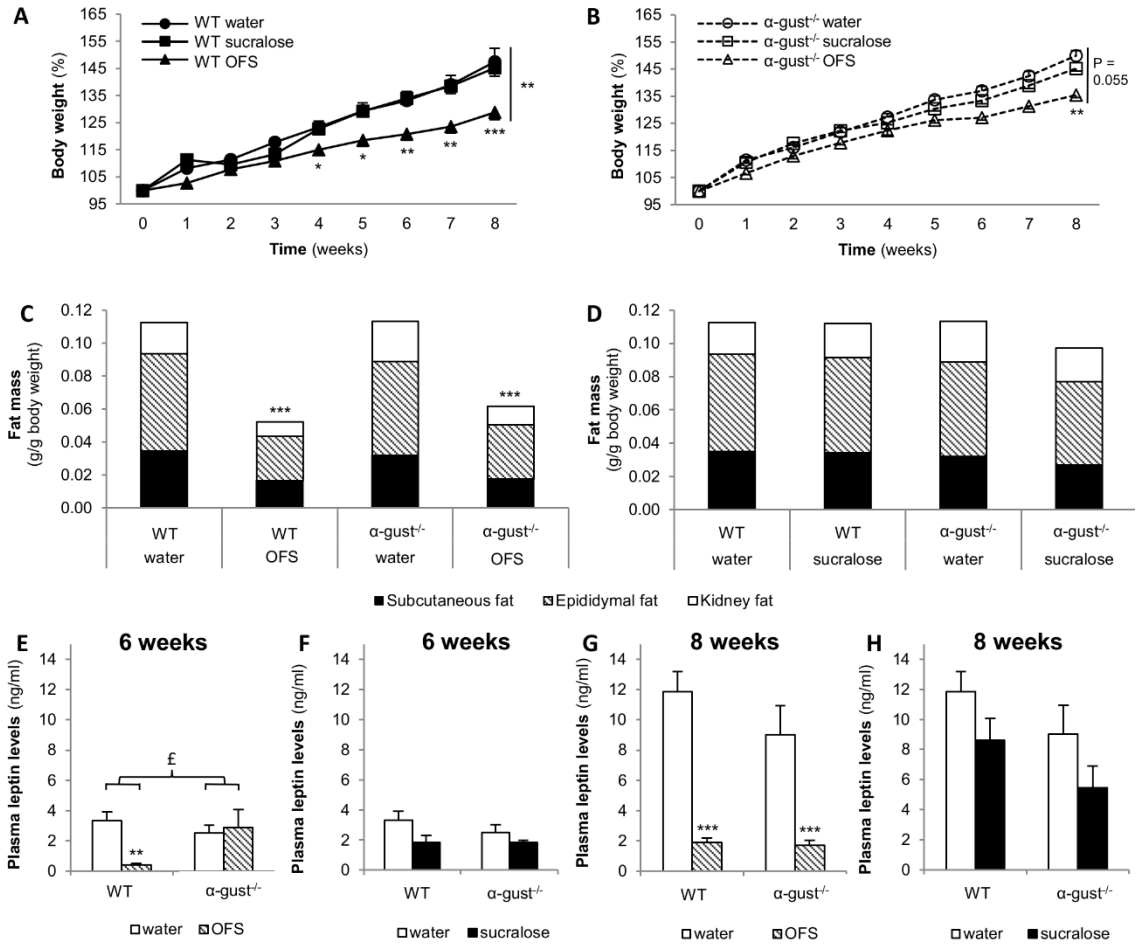


Figure 1: Natural, but not artificial sweeteners prevent HFD-induced body weight gain. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on (**A, B**) time-dependent changes in body weight (n=9/group), (**C, D**) fat mass (n=9/group) and (**E-H**) plasma leptin levels after 6 or 8 weeks (n=7-9/group) of treatment. Results are presented as mean±SEM. *P<0.05, **P<0.01 ***P<0.001 vs. control. Genotype*treatment effect: £ P<0.05.

Neither OFS nor sucralose administration affected cumulative food intake in either genotype (Figure S1A, B).

4.5.2 OFS, but not sucralose reduces HFD-induced insulin resistance in an α-gustducin-independent manner

6 weeks HFD impaired glucose tolerance to a similar extent in WT and α-gust$^{-/-}$mice (Figure 2A, B). OFS treatment did not affect blood glucose during the OGTT in either genotype, but decreased serum insulin profiles (P<0.05) and the AUC (P<0.01) during an OGTT in WT mice but not in α-gust$^{-/-}$mice (Figure 2A-D). The insulin resistance index (HOMA-IR) was lower in both genotypes (Figure 2E).

Sucralose treatment did not improve blood glucose during the OGTT in either genotype, but tended (P=0.057) to increase serum insulin profiles and significantly (P<0.01) increased insulin AUC values in a genotype-dependent manner in WT mice (treatment*genotype; P<0.05) (Figure 2A-D). The HOMA-IR was not affected in either genotype (Figure 2F).

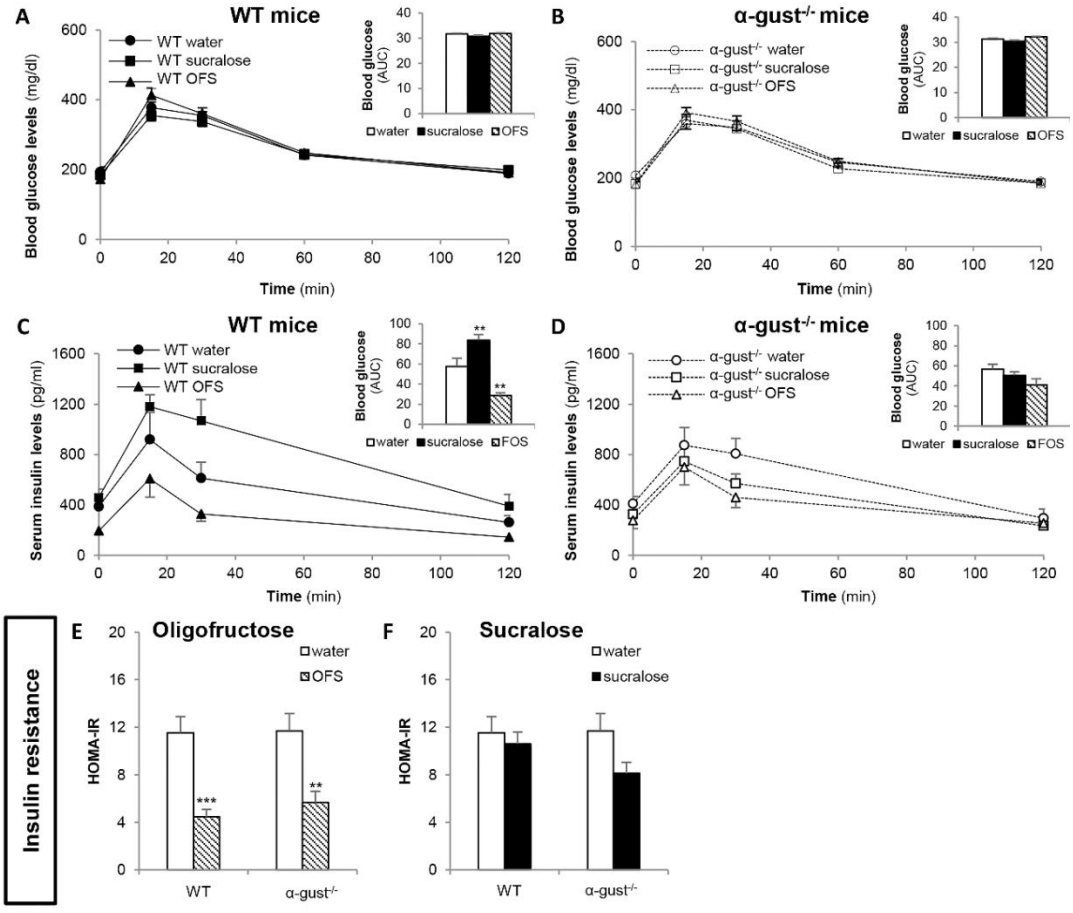


Figure 2: Intragastric administration of natural sweeteners prevent HFD-induced insulin resistance. An OGTT was performed in WT and α-gust$^{-/-}$mice (n=9) 6 weeks after daily gavage with OFS (300 mg), sucralose (0.52mg) or water. (**A, B**) Plasma glucose and (**C, D**) serum insulin profiles during an OGTT and the calculated AUC (n=7-9/group) and (**E, F**) calculated insulin resistance index (HOMA-IR). Results are presented as mean±SEM. **P<0.01, ***P<0.001 vs. control.

4.5.3 OFS, but not sucralose affects gut hormone secretion in an α-gustducin-independent manner

4.5.3.1 Ghrelin

OFS, but not sucralose, significantly increased plasma octanoyl ghrelin levels in α-gust$^{-/-}$mice ($P<0.05$), but not in WT mice (Figure 3A, B). A representative immunofluorescence staining for octanoyl ghrelin in gastric sections of all groups is shown in Figure 3C. OFS did not affect gastric ghrelin immunoreactive cell density but decreased ($P<0.01$) gastric ghrelin IR cell intensity in α-gust$^{-/-}$mice (Figure 3D, E). Neither sweetener affected gastric nor duodenal ghrelin mRNA expression levels (Figure 3F, G).

4.5.3.2 GLP-1

OFS decreased glucose-induced plasma GLP-1 (GLP-1(7-36)amide and GLP-1(7-37)) levels in WT mice ($P<0.001$), but not in α-gust$^{-/-}$mice. Notably, water-treated α-gust$^{-/-}$mice tended to have lower plasma GLP-1 levels ($P=0.056$) than water-treated WT mice (Figure 4A, B).

The origin of the plasma changes was determined by measuring the number of L-cells and the mRNA expression levels of proglucagon in the duodenum and colon. Despite the increase ($P<0.001$) in duodenal mucosal height in both genotypes after OFS administration (Figure S2A), the number or density of duodenal GLP-1 IR L-cells was not affected. Only duodenal GLP-1 IR L-cell intensity, which is a reflection of the amount of GLP-1 released, tended to be decreased ($P=0.054$) in WT but not in α-gust$^{-/-}$mice (Figure 4C-E). Moreover, duodenal proglucagon mRNA expression levels were decreased ($P<0.05$) in α-gust$^{-/-}$mice (Figure 4I). In the colon, mucosal height was specifically increased in WT ($P<0.001$) but not in α-gust$^{-/-}$mice (treatment*genotype; $P<0.05$) after OFS administration (Figure S2B). This did not result in changes in colonic GLP-1 IR L-cell number, density or intensity. Colonic proglucagon mRNA levels were not affected in either genotype (Figure 4F-I).

Sucralose tended ($P=0.054$) to increase glucose-induced plasma GLP-1 (GLP-1 (7-36)amide and GLP-1 (7-37)) levels in α-gust$^{-/-}$mice but not in WT mice (Figure 4B). Sucralose also did not alter duodenal or colonic mucosal height in either genotype (Figure S2C, D) and did not affect duodenal or colonic GLP-1 IR L-cell number, density, intensity (data not shown) or proglucagon mRNA expression levels (Figure 4J).

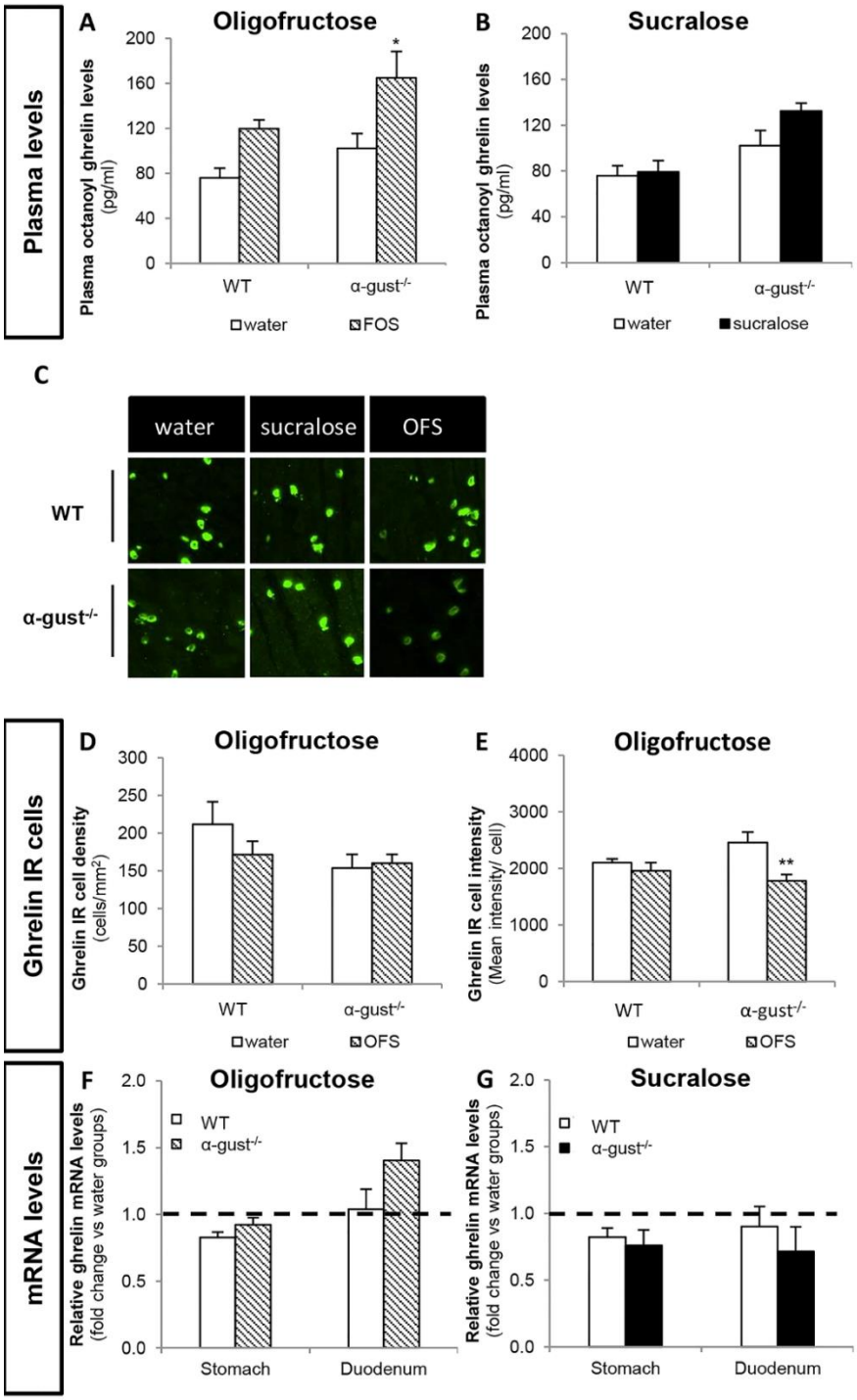


Figure 3: Natural sweeteners increase plasma ghrelin levels in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on (**A, B**) glucose-induced plasma octanoyl ghrelin levels (n=7-9/group), (**C**) Representative immunofluorescence staining for octanoyl ghrelin in stomach sections of WT and α-gust$^{-/-}$mice. (**D, E**) density and mean intensity of gastric octanoyl ghrelin IR cells (n=5/group) and (**F, G**) relative gastric and duodenal ghrelin mRNA levels (n=7-8/group). The dotted line indicates the mean relative ghrelin mRNA levels in controls. Results are presented as mean±SEM. *P<0.05, **P<0.01 vs. control.

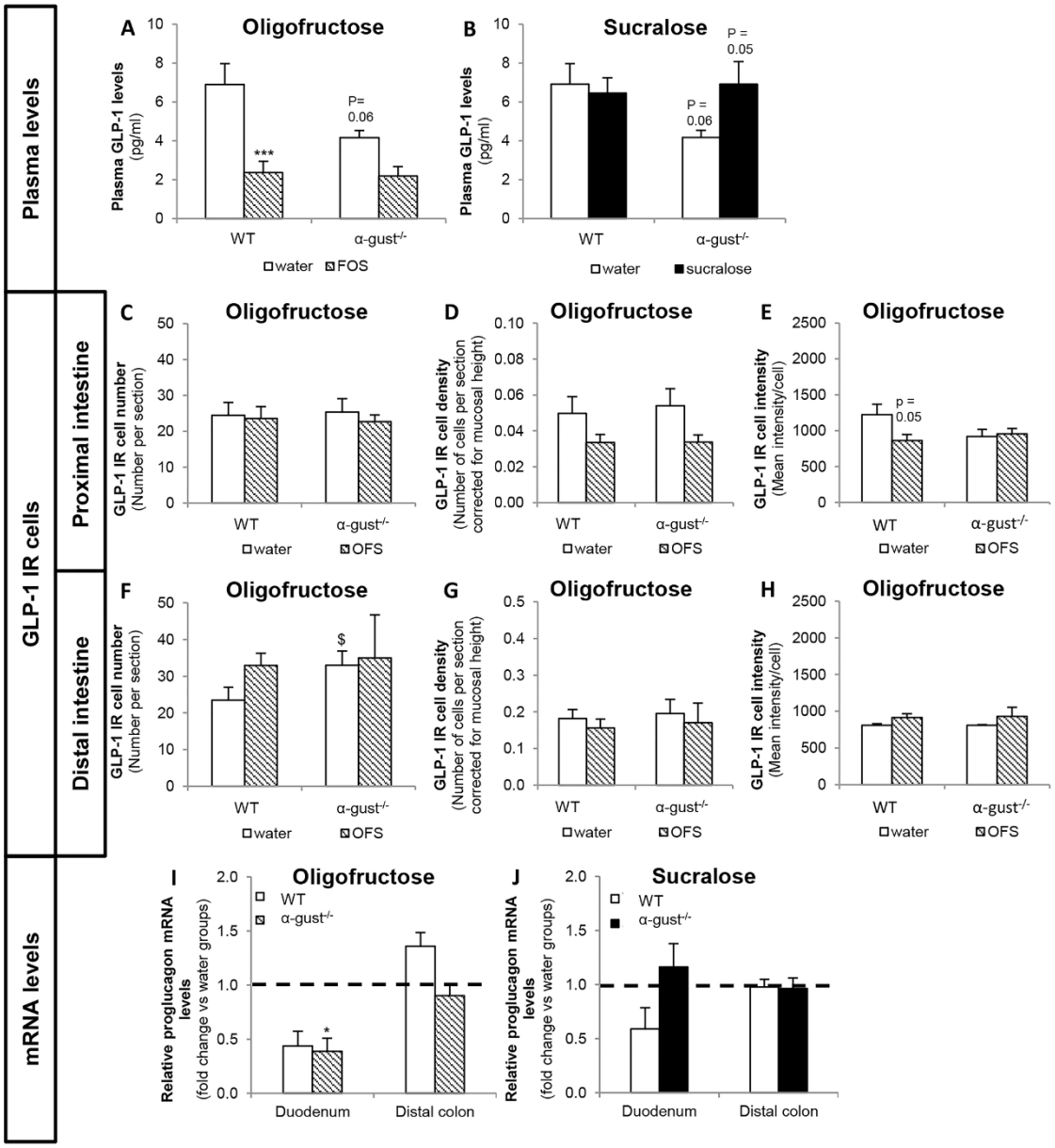


Figure 4: Natural sweeteners decrease plasma GLP-1 levels in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on (**A, B**) glucose-induced plasma levels of GLP-1(7-36)amide and GLP-1(7-37) (**C, F**) number, (**D, G**) density and (**E, H**) mean intensity of duodenal and colonic GLP-1 IR cells (n=4-5/group) and (**I, J**) relative duodenal and colonic proglucagon mRNA levels (n=8-9/group). The dotted line indicates the mean relative proglucagon mRNA levels in controls. Results are presented as mean±SEM. * P<0.05, *** P<0.001 vs. control. $ P<0.05 vs. the water-treated WT group.

4.5.3.3 PYY

OFS administration did not affect glucose-induced plasma PYY (PYY(1-36) and PYY(3-36)) levels in either genotype (Figure 5A, B). However, sucralose administration tended (P=0.056) to increase PYY levels in WT mice. Neither of the sweeteners changed the number or intensity of the colonic PYY IR L-cells (Figure 5C, D). The increased colonic mucosal height after OFS administration resulted in a decreased PYY IR L-cell density in WT (P<0.01), but not in α-gust$^{-/-}$ mice (treatment*genotype; P<0.01)(Figure 5E). Colonic PYY mRNA levels were not affected (Figure 5F, G).

In water-treated WT mice, 48% of the L-cells selectively stained for GLP-1 and 59% for PYY. Treatment with the sweeteners did not induce a shift in the L-cell subpopulations (OFS: 63% GLP-1, 46% PYY; sucralose: 46% GLP-1, 42% PYY).

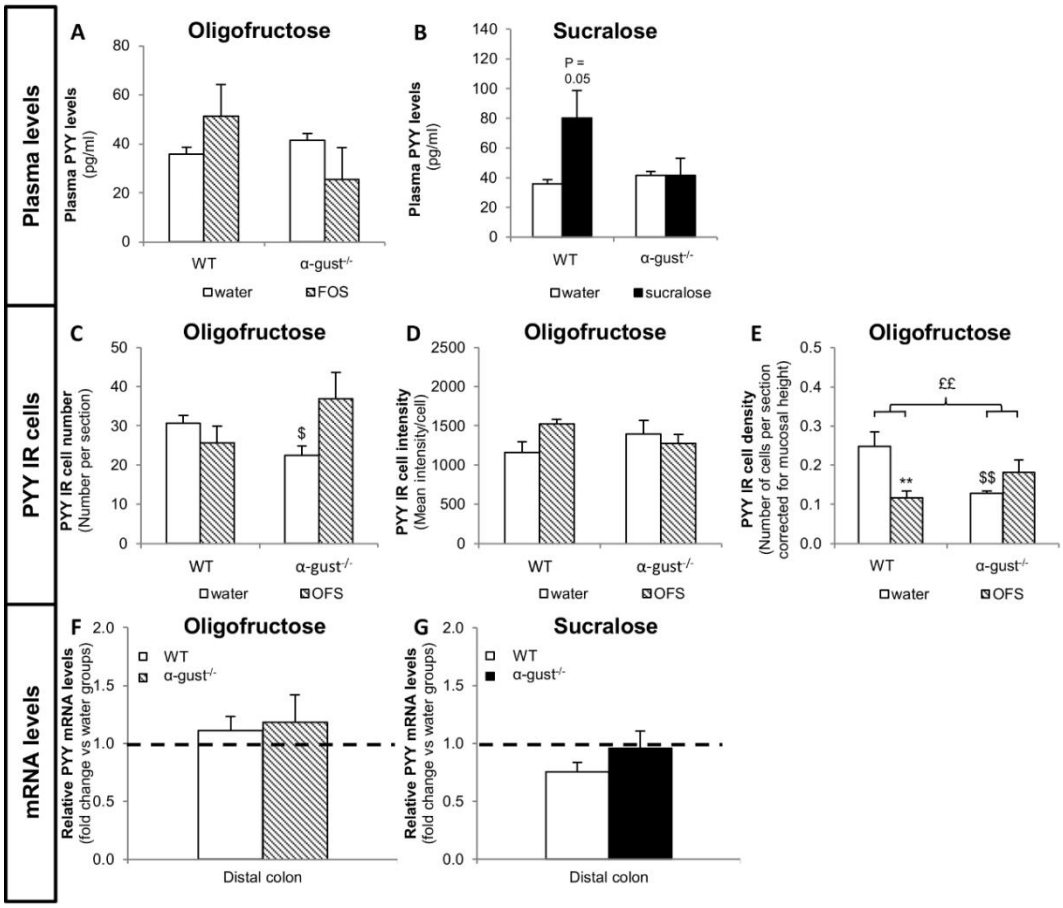


Figure 5: Natural nor artificial sweeteners affect plasma PYY levels in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on (**A, B**) glucose-induced plasma levels of PYY(1-36) and PYY(3-36) (n=7-8/group), (**C**) number, (**D**) density and (**E**) mean intensity of colonic PYY IR cells (n=5/group) and (**F, G**) relative colonic PYY mRNA levels (n=7-8/group). The dotted line indicates the mean relative PYY mRNA levels in controls. Results are presented as mean±SEM. ** P<0.01 vs. control. $ P<0.05, $$ P<0.01 vs. the water-treated WT group. Genotype*treatment effect: ££ P<0.01.

4.5.4 OFS, but not sucralose affects the expression of nutrient sensors at local and remote sites

4.5.4.1 Effect on the expression of glucose sensors in the proximal gut

Neither sweetener changed relative gastric TAS1R3 mRNA levels, a subunit of the sweet TASR heterodimer (Figure 6A, B). The gastric mRNA expression of the other sweet TASR subunit, TAS1R2, and the glucose transporters SGLT1 and GLUT2 was undetectable. Furthermore, neither sweetener altered duodenal TAS1R2 or TAS1R3 mRNA levels. However, OFS but not sucralose administration significantly decreased duodenal mRNA expression of SGLT1 and GLUT2 in both genotypes (Figure 6C, D).

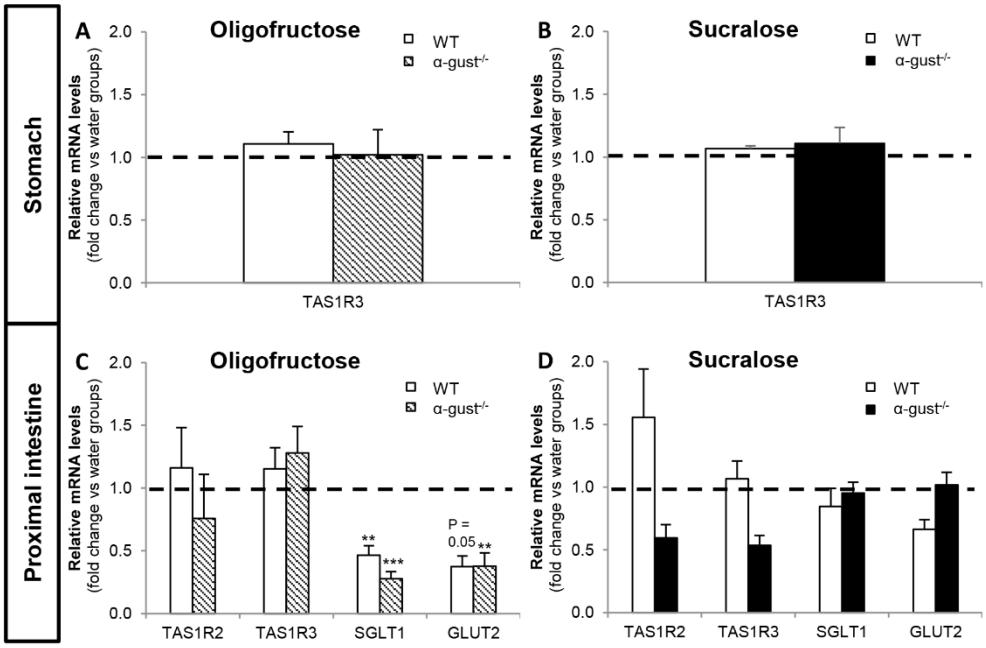


Figure 6: Natural, but not artificial sweeteners affect the glucose transporters in the proximal gastrointestinal tract in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on relative (**A, B**) gastric and (**C, D**) duodenal mRNA levels of the sweet TASR subunits (TAS1R2 and TAS1R3) and the monosaccharide transporters (SGLT1, GLUT2) (n=7-9/group). The dotted line indicates the mean relative mRNA levels in controls. Results are presented as mean±SEM. ** P<0.01, *** P<0.001 vs. control.

4.5.4.2 Effect on the SCFA production and signaling in the distal gut

The wet caecal weight was higher in OFS-treated mice compared to controls (WT:203%; α-gust$^{-/-}$:353%) but not after sucralose treatment (WT:93%; α-gust$^{-/-}$:124%).

Furthermore, OFS administration decreased caecal levels of the microbial fermentation products; acetate and butyrate but not propionate in both genotypes (Figure 7A-C). These decreased levels may result from an increased caecal uptake by the SCFA transporter monocarboxylate transporter-1 (MCT1). OFS treatment significantly downregulated MCT1 mRNA expression in α-gust$^{-/-}$mice, but not in WT mice (P=0.08) (Figure 7G).

In contrast, sucralose did not affect acetate or butyrate levels, but increased (P<0.05) propionate levels in WT but not in α-gust$^{-/-}$mice (Figure 7D-F). Caecal MCT1 mRNA expression was not affected in either genotype (Figure 7H).

SCFAs directly interact with FFAR2/3 in the colon to affect GLP-1 and PYY secretion (Nohr et al., 2013). OFS decreased colonic FFAR3 (P<0.001), but not FFAR2 mRNA levels in both genotypes (Figure 7G). Sucralose did not affect FFAR2 mRNA levels, but surprisingly decreased (P<0.001) FFAR3 mRNA levels in α-gust$^{-/-}$mice, but not in WT mice (Figure 7H).

4.5.4.3 Effect on the expression of SCFA receptors in the proximal gut

After uptake, SCFAs enter the blood stream and interact with FFAR2/3 expressed in remote tissues. OFS, but not sucralose administration decreased duodenal FFAR2 expression in α-gust$^{-/-}$mice (P<0.05), but not in WT mice, and tended to decrease FFAR3 mRNA levels in both genotypes (Figure 7I, J). In contrast, neither sweetener affected gastric FFAR2/3 expression (Figure 7I, J).

4.5.4.4 Effect on the expression of SCFA receptors in adipose tissue

SCFAs affect adipocyte differentiation via FFAR2-dependent and independent pathways (PPARγ) (Δεωυλφ ετ αλ., 2011). Neither OFS nor sucralose affected PPARγ mRNA expression levels (Figure 7K, L). In contrast OFS, but not sucralose, increased (P<0.05) FFAR2 expression in subcutaneous fat in WT, but not in α-gust$^{-/-}$mice (Figure 7K, L). Subcutaneous fat FFAR3 mRNA levels were not detectable.

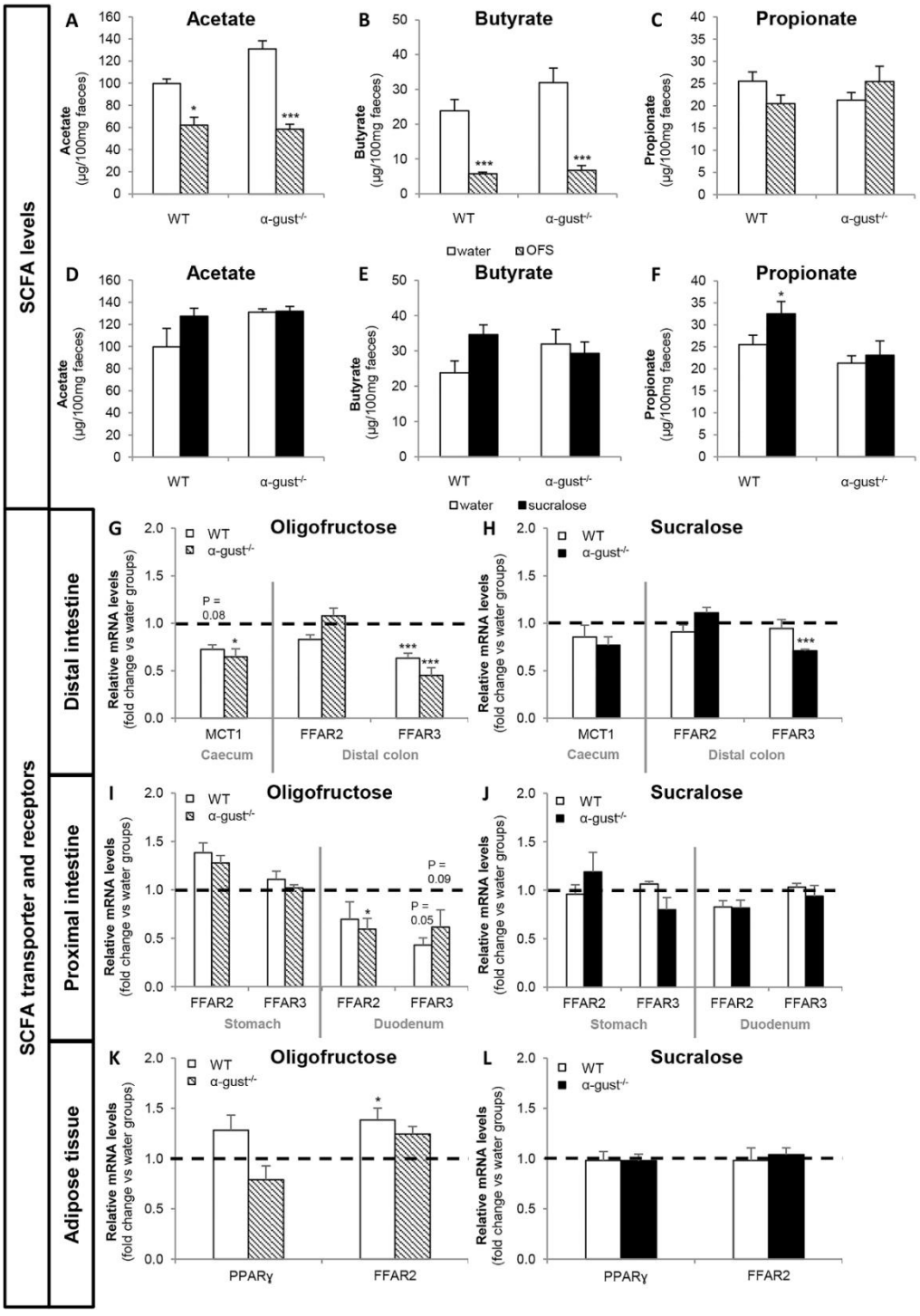


Figure 7: Natural and artificial sweeteners affect SCFA production and FFAR2/3 expression in the proximal and distal gastrointestinal tract in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on caecal (**A, D**) acetate, (**B, E**) butyrate and (**C, F**) propionate levels (n=7-9/group), relative mRNA levels of (**G, H**) caecal MCT1 and colonic FFAR2 and FFAR3, (**I, J**) gastric and duodenal FFAR2 and FFAR3 and (**K, L**) PPARɣ and FFAR2 in subcutaneous fat (n=7-9/group). The dotted line indicates the mean relative mRNA levels in controls. Results are presented as mean±SEM. * P<0.05, *** P<0.001 vs. control.

4.5.4.5 OFS, but not sucralose improves gut permeability in an α-gustducin-independent manner

Sucralose did not affect gut permeability, but OFS increased TEER (P<0.001) and decreased fluorescein passage (P<0.001) in both genotypes (Figure 8A-D).

After correcting for the α-gustducin-dependent colonic hypertrophy, the increase in TEER (Figure 8C, D) and decrease in fluorescein passage (data not shown) were more pronounced in both genotypes. Furthermore the corrected TEER, but not the corrected fluorescein passage, became α-gustducin-dependent and more pronounced in WT mice (treatment*genotype; P<0.05) (Fig 8C). OFS but not sucralose decreased (WT: P<0.05; α-gust$^{-/-}$: P<0.01) the expression of claudin 5, a tight junction protein, independent of the genotype (Figure 8E, F).

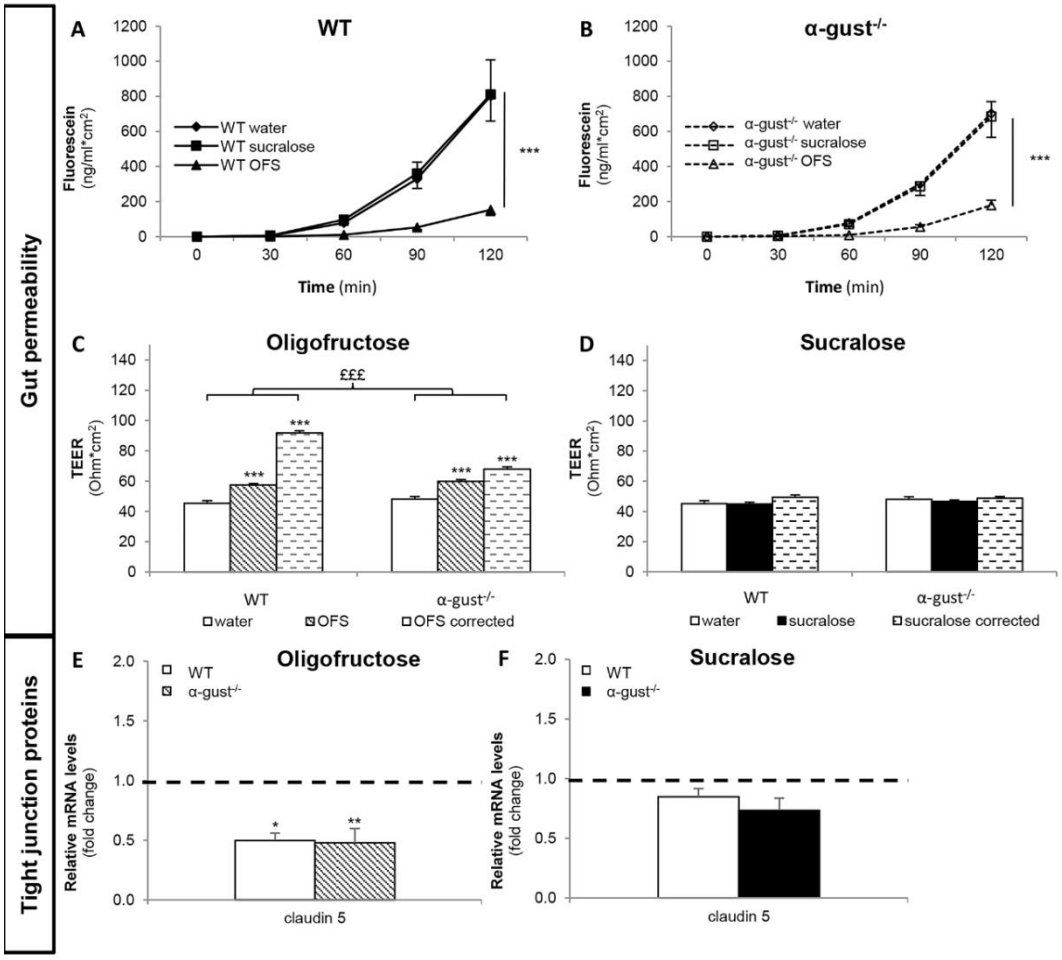


Figure 8: Natural, but not artificial sweeteners improve gut permeability in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on (**A, B**) colonic fluorescein passage (n=6-9/group), (**C, D**) transepithelial electrical resistance (TEER) and TEER corrected for mucosal height (n=7-8/group) and (**E, F**) relative colonic claudin 5 mRNA expression levels (n=7-9/group). The dotted line indicates the mean relative claudin 5 mRNA levels in controls. Results are presented as mean±SEM. *P<0.015, **P<0.01, ***P<0.001 vs. control. Genotype*treatment effect: £££ P<0.001.

4.6 Discussion

Low caloric sweeteners could be used as food additives to favor body weight loss. These sweeteners may not be metabolically inert since they can affect the release of gut hormones involved in the control of energy –and glucose homeostasis through modulation of the nutrient sensing pathway. To investigate this hypothesis we determined whether daily intragastric administration of equisweet amounts of low caloric non-fermentable (sucralose) or fermentable (OFS) sweeteners can prevent obesity-associated disorders by interacting with extra-oral taste receptors specifically coupled to α-gustducin to alter gut hormone release.

Our data show that long-term *in vivo* supplementation with sucralose did not modulate nutrient sensor expression, gut hormone release, intestinal permeability, glucose homeostasis or body weight, indicating that sweet taste receptor activation does not play an important regulatory role in these parameters. In contrast, intragastric administration of OFS decreased body weight gain and fat pad mass in mice. This was not mediated via gustducin or the result of an altered energy intake or gut hormone release since GLP-1 levels were decreased. Rather enhanced uptake of OFS fermentation products (acetate, butyrate) favored interaction with FFAR2 in peripheral adipose tissue to reduce adipogenesis. Furthermore OFS did not improve glucose intolerance but reduced the development of insulin resistance and improved the increased gut permeability induced by a HFD.

OFS but not sucralose reduced body weight gain. This effect was delayed in α-gust$^{-/-}$mice, and was reflected in a retarded reduction in plasma leptin levels. This suggests that α-gustducin plays a role in the delayed formation of fat deposits after OFS supplementation. α-gustducin has previously been shown to play a role in brown adipose tissue thermogenic activity (Avau et al., 2015). The decreased body weight gain was not reflected in differences in energy intake which confirms previous observations in mice fed a HFD supplemented with OFS (Dewulf et al., 2011).

Intragastric OFS administration did not improve glucose intolerance. A systematic review also showed no significant effect of inulin-type fructans on glucose levels in humans (Bonsu et al., 2011). Our findings and previous mice studies suggest that the route of administration determines the outcome of OFS-supplementation. Indeed, 1) a HFD supplemented with 10% OFS (=±300mg/day) improved glucose homeostasis and body weight (Cani et al., 2006), 2) supplementation of the drinking water with 300mg OFS/day improved glucose homeostasis but not body weight (Everard et al., 2011), and 3) intragastric administration of 300mg OFS/day, in our study, improved body weight but not glucose homeostasis. The intragastric

administration allowed us to administer a fixed amount at a well determined time point to mimic the pharmacological profile patients would get after a single supplement before a meal. Furthermore, it allowed us to bypass potential participation of the cognitive behavioral axis since sweet taste receptors are also present on the tongue and play a role in hedonic signals.
Sucralose neither affected glucose tolerance nor insulin resistance although it increased serum insulin levels during an OGTT in an α-gustducin-dependent manner. Sucralose has also been shown to increase glucose-induced insulin levels associated with an increase in peak plasma glucose concentrations in obese subjects after an acute administration (Pepino et al., 2013). Previous studies showed that sucralose induced insulin release from the pancreatic beta cell line, MIN6. In contrast to our findings, knockdown of gustducin in this model did not lead to an attenuated insulin release in the presence of three sweeteners (Nakagawa et al., 2013). These results contradict the hypothesis of a direct involvement of gustducin on pancreatic beta cells. Nevertheless, the sweet taste receptors and short chain fatty acid receptors are present on pancreatic beta cells, suggesting a role for nutrient sensing in insulin release (Nakagawa et al., 2009, Regard et al., 2007).

Neither OFS nor sucralose affected plasma levels of the orexigenic and adipogenic hormone ghrelin in WT mice. It is therefore unlikely that the reduced body weight after OFS supplementation is mediated by ghrelin. Previous rodent studies also showed no effect on plasma ghrelin levels after supplementation of a western style diet with 10% OFS for 6-7 weeks (Cluny et al., 2015). Although others reported decreased plasma ghrelin levels after 3 weeks of a standard diet enriched with 100g/day OFS (Cani et al., 2004a). Surprisingly OFS administration increased glucose-induced plasma ghrelin levels in α-gust$^{-/-}$mice but the physiological relevance is unclear.
One of the limitations of our study is that we could only measure gut hormone levels at a single time point. Although plasma GLP-1 levels peak 10 min after glucose administration, this time-point is too early to observe a postprandial decline in plasma ghrelin levels. Therefore the measured levels rather represent fasted plasma ghrelin levels. Tarini *et al*. observed a delayed (5h) effect of inulin administration on ghrelin levels, indicating that the decrease in plasma ghrelin levels parallels the start of the colonic fermentation (Tarini and Wolever, 2010).

The decreased plasma levels of the satiety hormone GLP-1 in WT mice after OFS administration can also not explain the reduced body weight, but could explain why OFS decreased glucose-induced insulin levels in WT mice (decreased incretin effect) and was unable to improve glucose intolerance in our study. Indeed, previous studies in GLP-1R$^{-/-}$ mice or administration of the GLP-1 receptor antagonist exendin 9-39 totally prevented the

beneficial effects of OFS on glucose intolerance and body weight gain (Delzenne et al., 2007, Cani et al., 2006). In healthy volunteers and in overweight and obese subjects, 5-6 weeks of OFS supplementation did not alter plasma GLP-1 levels (Pedersen et al., 2013, Daud et al., 2014).

The decrease in plasma GLP-1 levels probably originated from changes in the duodenum. Indeed, proglucagon expression and GLP-1 IR L-cell intensity were decreased in the duodenum together with SGLT1 and GLUT2 mRNA expression in WT mice. These glucose transporters have been shown to function as glucose sensors for the L-cells and could contribute to the reduced duodenal proglucagon expression and hence GLP-1 release (Gorboulev et al., 2011, Reimann et al., 2008). Furthermore, the decreased expression of SGLT1 and GLUT2 could result in a decreased glucose transport from the lumen into the blood stream. However, no differences in peak glucose levels were observed between the different treatment and the different groups. Sucralose administration did not affect glucose-induced GLP-1 release. Artificial sweeteners have been shown to elicit GLP-1 release *in vitro* (Jang et al., 2007, Reimann et al., 2008) These findings were not confirmed *in vivo* in humans and rodents and thereby question the importance of the sweet taste receptor complex in L-cells (Fujita et al., 2009, Steinert et al., 2011a) However, the 'equisweet' nature of the administered doses are based on human studies and the relative sweetness may differ in mice. Nevertheless we would like to point out that the expression pattern of sweet taste receptors and FFARs is quite similar between mice and man (Symonds et al., 2015).

Sucralose, but not OFS administration tended to increase plasma PYY levels in WT animals. In contrast, studies in rodents and humans observed an increased plasma PYY levels after OFS supplementation (Pedersen et al., 2013, Daud et al., 2014), while a single dose of artificial sweeteners did not affect plasma PYY levels in a double blinded cross-over study (Steinert et al., 2011a).

OFS is fermented by the gut microbiome to SCFAs that interact with FFAR2 or FFAR3 on EECs. The decrease in caecal acetate and butyrate levels could be due to an increased SCFA uptake by the MCT1 transporter on the colonocytes which displayed a compensatory downregulation. Sucralose increased caecal propionate levels. This is in agreement with a recent study of Suez *et al.,* who showed that artificial sweeteners consumption functionally altered the microbiota leading to elevated stool SCFA levels (Suez et al., 2014).

SCFAs can affect body weight and adiposity. In humans, targeted colonic delivery of an inulin-propionate ester was more effective in decreasing body weight than inulin alone, without affecting plasma GLP-1 and PYY levels (Chambers et al., 2015). Clearly, the increased propionate after sucralose administration was not high enough to elicit these effects in our

study. SCFAs can stimulate GLP-1 and PYY secretion from L-cells *in vitro* and *in vivo* in a FFAR2, and FFAR3 dependent manner (Tolhurst et al., 2012). Although both sweeteners decreased colonic FFAR3 mRNA levels, neither sweetener affected the colonic PYY or GLP-1 containing L-cell number or intensity, nor the PYY or proglucagon mRNA levels. Therefore a direct effect of SCFAs via FFARs on EECs of the distal colon was not shown. Although plasma SCFA levels could not be quantified due to our study design, we assume that SCFA levels increased in the blood stream after MCT1-mediated transport and can interact with FFAR2/3 receptors in other peripheral tissues. FFAR2/3 transcripts are found on both the apical and basolateral side of L-cells in the proximal intestine (Nohr et al., 2013) and FFAR2 is found in adipocytes (Dewulf et al., 2011).

Since duodenal FFAR2 mRNA levels were decreased after OFS, we speculate that circulating SCFAs might be involved in the decreased plasma GLP-1 levels. Surprisingly these effects occurred in a genotype-independent manner although Li. *et al*. showed that the SCFAs affect GLP-1 release in a α-*gus*tducin-dependent manner, at least in the colon (Li et al., 2013).

After entering the bloodstream the SCFAs can also directly affect the adipose tissue via FFAR2-dependent or FFAR2-independent pathways involving PPARγ (Dewulf et al., 2011). However, neither of the sweeteners affected PPARγ mRNA expression. In contrast, OFS supplementation increased FFAR2 mRNA levels in the adipose tissue, which could point towards an FFAR2-dependent pathway. The genotype-independent nature of the decreased fat mass would suggest that FFAR2 is coupled to another G-protein in the adipose tissue. Indeed, FFAR2 may activate the G_i, G_q, and G_{12} families of G proteins (Brown et al., 2003).

OFS, but not sucralose supplementation, decreased the colonic permeability for ions and larger molecules via an α-gustducin-independent mechanism. This improvement in colonic permeability was not mediated by changes in mucosal height. It has previously been demonstrated that OFS supplementation alters tight junction expression and thereby decreases gut permeability (Cani et al., 2009). Claudin 5 is a tight junction protein which influences the paracellular leak passage of small molecules of approximately 800 Da (Gunzel and Yu, 2013). OFS decreased claudin 5 mRNA expression independent of α-gustducin which could explain the decreased gut permeability if the downregulation is a reflection of increased protein expression (Weber, 2012). Strangely the improved permeability did not result in lower glucose profiles, although the link between intestinal permeability and glucose homeostasis has been shown previously (Cani et al., 2009).

In conclusion, despite the controversy in the field our study shows that the artificial sweetener, sucralose, is metabolically inert. Nevertheless, oligofructose, an oligosaccharide

with prebiotic properties has beneficial effects on body weight gain. The dose used in these mice is relatively high and unlikely to be used in humans because of flatulence, bloating, cramps and abdominal pain due the increase gas production associated with fermentation. However, agonists targeting FFAR2/3 might be an alternative but the route and time point of administration may be important.

Alterations in gut hormone profiles are not mandatory for the beneficial effect of oligofructose on body weight. Our data rather suggest that targeting of FFAR2 receptors on adipose tissue might be the way forward to combat obesity. However, these results should first be verified in human intervention studies. Furthermore, our results highlight that gustducin-mediated nutrient signaling does not play an important role in the effects of oligofructose on energy homeostasis although FFAR2/3 signaling through a different G protein might contribute to these effects and needs to be addressed in additional studies

Author contributions: Conceptualization, S.S. and I.D.; Formal Analysis, S.S., L.C.; Investigation, S.S., L.C., T.T, B.A. and L.V.; Writing – Original Draft, S.S. and I.D.; Writing – Review & Editing, S.S., L.C., T.T., B.A., L.V., R.F., K.V. and I.D.; Funding Acquisition S.S. and I.D.; Resources, R.F., K.V. and I.D.; Supervision, S.S. and I.D.

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4.7 Expanded Materials and Methods

4.7.1 Oral glucose tolerance test

An OGTT was performed 6 weeks after the start of the experiment. Mice were fasted for 6 hours and gavaged with 2g/kg D-glucose. Blood glucose levels were measured from the tail vein at 0, 15, 30, 60 and 120 minutes after glucose administration with a glucometer (Glucomen LX®, A. Menarini Diagnostics) and serum was collected and subsequently snap frozen in liquid nitrogen for insulin and leptin measurements. Insulin resistance was calculated using the Homeostasis Model of Insulin Resistance (HOMA-IR) index: (fasting insulin (ng/mL) × fasting glucose (mg/dL))/405.

4.7.2 Plasma ghrelin measurement

Plasma samples were extracted on a SEP-Pak C18 cartridge (Waters Corporation), vacuum-dried and subjected to ghrelin RIA as previously described (Janssen et al., 2011). For determination of octanoyl ghrelin a rabbit anti-human ghrelin [1-8] antibody was used which does not recognize desoctanoyl ghrelin. Total ghrelin levels were determined using a rabbit anti-human ghrelin [14-28] antibody, which recognizes both octanoyl and desoctanoyl ghrelin.

4.7.3 Plasma Glucagon-Like Peptide-1, Peptide YY, Insulin and Leptin measurement

Samples were analysed for plasma GLP-1 (GLP-1(7-36)amide and GLP-1(7-37)) using the active GLP-1 (ver. 2) Kit (Mesoscale Discovery) and plasma leptin and serum insulin levels were determined using the Mouse metabolic Kit (Mesoscale Discovery). Plasma PYY levels (PYY(1-36) and PYY(3-36)) were determined using a PYY ELISA (Phoenix pharmaceuticals).

4.7.4 Histology and Immunohistochemistry

Duodenal and colonic segments (8 µm), were stained with hematoxylin and eosin. The mucosal villus height was determined by counting five representative high-power fields (20x) per slice (3 slices/animal). Gastric, duodenal or colonic tissue samples were immediately fixed with paraformaldehyde (4%) for 2h (4°C) followed by cryoprotection in 30% sucrose at 4°C overnight. Cryostat sections (8 µm) were incubated for 2 h in 0.1M PBS containing 10% donkey serum and 0.3% Triton X-100 and incubated with one of the following primary antibodies: rabbit anti-octanoyl ghrelin (1/1000, Ab5004, in-house developed), goat anti-GLP-1 (1/150, SC-7782, Santa Cruz Biotechnology) or rabbit anti-PYY (1/1000, Ab22663, Abcam). Substitution of the primary antibody with phosphate-buffered saline was used as a negative control. After washing, tissues were incubated with secondary antibody for 2 hours: donkey anti-rabbit Alexa488 (1/1500, A21206, Thermofisher), anti-goat Alexa594 (1/1000, A11058, Thermofisher) or donkey anti-rabbit AMCA (1/250, 711-225-152, Jackson Immuno Labs). For the double-immunofluorescence staining, after being incubated with the secondary antibody and washed three times, sections were incubated for 24 hours at room

temperature with the second primary antibody. Subsequently, tissues were incubated with the appropriate second secondary antibody for 2 hours. Sections were mounted in Citifluor and visualized under a fluorescence microscope (Olympus BX41). Of each tissue segment three sections were analyzed using Cell^F Imaging Software (Olympus Soft Imaging Solutions GmbH). Octanoyl ghrelin-, GLP-1- or PYY-positive cells, were counted in 5 randomly chosen fields (20x) for the stomach, or in the whole section (20x) for the segments from the duodenum and distal colon.

4.7.5 RT-PCR

Total RNA was isolated from tissue segments using the RNeasy kit (Qiagen),treated with Turbo DNAfree kit (Ambion) and reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen). The qRT-PCR reaction was performed as described previously, using the Lightcycler 480 (Roche Diagnostics) with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics) (Verhulst et al., 2008), and analyzed according to the method of Vandesompele *et al*. (Vandesompele et al., 2002). Results were expressed relative to the geometric mean of the normalized expression of the three most stable housekeeping genes tested; hypoxanthine phosphoribosyltransferase 1 (Hprt1), ribosomal protein L13a (RPL13a) and β-actin.

The following primers were used:

Gene	**Forward primer**	**Reverse primer**
RPL13a	CACTCTggAggAgAAACggAAgg	gCAggCATgAggCAAACAgTC
β-Actin	gATCTggCACCACACCTTCTAC	TggATggCTACgTACATggCTg
Hprt1	TCAgTCAACgggggACATAAA	ggggCTgTACTgCTTAACCAg
ghrelin	CCAgAggACAgAggACAAgC	ACATCgAAgggAgCATTgAA
GOAT	ATTTgTgAAgggAAggTggAg	CAggAgAgCAgggAAAAAgAg
proglucagon	gAggAgAACCCCAgATCATTCC	gTggCgTTTgTCTTCATTCATC
PYY	CAgTggTgAAgACTCCCCCAAg	TgAACACACACAgCCCTCCAg
SGLT1	CggAAgAAggCATCTgAgAA	AATCAgCACgAggATgAACA
GLUT2	TCTTCACggCTgTCTCTgTg	AATCATCCCggTTAggAACA
TAS1R2	gCACCAAgCAAATCgTCTATCC	ATTgCTAATgTAggTCAgCCTCgTC
TAS1R3	CAggCAgTTgTgACTCTgTTg	TgCgATgCAgATACCTCgTg
FFAR2	CCCTgTgCACATCCTCCTgC	gCgTTCCATgCTgATgCCCg
FFAR3	TgTCCAATACTCTgCATCTgT	AggTCCgAAATggTCAggTT
Claudin 5	gCATCCTgCTggggCTgATCg	ggCTTgggATAAggCCgTggTg

4.7.6 SCFA analysis

SCFAs were extracted from fecal samples using an ether extraction. 50 mg of fecal sample was suspended in 1 ml saturated NaCl (36%). Ether (3 ml), Na_2SO_4 (50 mg), H_2SO_4 (150 µl) and internal standard (50 µl) (2-ethylbutyric acid (Merck)) was added to each sample. The ether layer was collected and analyzed on a GC-MS quadrupole (Finnigan Trace GC, Thermoquest) as previously described (De Preter et al., 2009). Samples were absolutely quantified using an calibration curve.

4.7.7 Gut Permeability Assays

The distal colon was dissected in carbogenated Krebs-Ringer buffer (11mM D-glucose). Intestinal segments of approximately 0.5 cm^2 (n=3/mouse) were mounted in Ussing chambers (Mussler Scientific Instruments) with an area of 0.017cm^2. Potential difference (PD) was continuously recorded and transepithelial electrical resistance (TEER) ($\Omega x cm^2$) was calculated from the voltage deflections induced by bipolar constant-current pulses of 16 mA every 60 s with duration of 200 ms and was recorded over 2h. Meanwhile, paracellular permeability was measured after a equilibration period of 30 minutes, by adding fluorescein (354 Da, Sigma Aldrich) to the mucosal side. The fluorescence level at the serosal side (ng/ml*cm^2) was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech).

4.8 Supplementary Figures

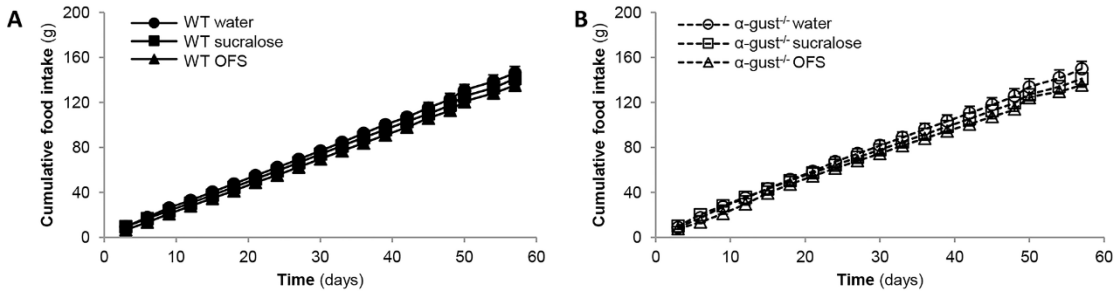

Figure S1: Natural and artificial sweeteners do not affect food intake in mice on a HFD. Effect of daily oral gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-$gust^{-/-}$mice on (**A, B**) time-dependent changes in cumulative food intake (n=9/group). Results are presented as mean±SEM.

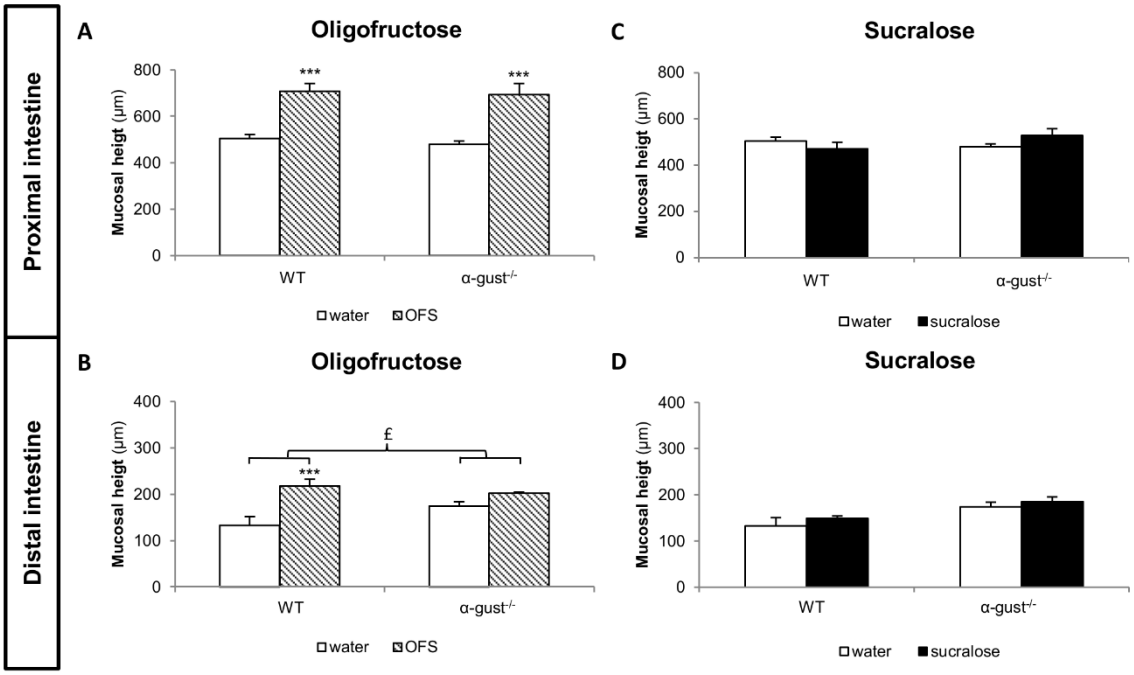

Figure S2: Natural sweeteners induce morphological changes in the proximal and distal gastrointestinal tract in mice on a HFD. Effect of daily gavage (8 weeks) of OFS (300mg), sucralose (0.52mg) or water in WT and α-gust$^{-/-}$mice on mucosal height of (**A, B**) duodenal and (**E, F**) colonic sections (8 µm) (n=5/group). Results are presented as mean±SEM. ***P<0.001 vs. control. Genotype*treatment effect: £ P<0.05.

Chapter 5

EXPLORING THE ROLE OF NUTRIENT SENSING IN THE METABOLIC CHANGES AFTER ROUX-EN-Y GASTRIC BYPASS (RYGB) SURGERY

The data represented in the following chapter are published in Journal of Endocrinology:

The role of nutrient sensing in the metabolic changes after gastric bypass surgery

Steensels S., Lannoo M., Avau B., Laermans J., Vancleef L., Farré R., Verbeke K., Depoortere I.

Journal of Endocrinology, **2017,** 232(3):363-376.

5 EXPLORING THE ROLE OF NUTRIENT SENSING IN THE METABOLIC CHANGES AFTER ROUX-EN-Y GASTRIC BYPASS (RYGB) SURGERY

5.1 Abstract

Taste receptors coupled to the gustatory G-protein, gustducin, on enteroendocrine cells sense nutrients to regulate gut hormone release. During Roux-en-Y gastric bypass (RYGB) surgery, the altered nutrient flow to more distal regions can affect gustducin-mediated gut hormone release and hence energy –and glucose homeostasis.

We studied the role of gustducin-mediated signaling in the metabolic improvements and intestinal adaptations along the gut after RYGB surgery in wild type (WT) and α-gustducin$^{-/-}$ (*α-gust*$^{-/-}$) mice.

RYGB surgery decreased body weight in WT and *α-gust*$^{-/-}$mice, while food intake was only decreased in WT mice. Pair-feeding to the RYGB group improved glucose homeostasis to a similar extent in WT mice. GLP-1 levels were increased in both genotypes, PYY levels in *α-gust*$^{-/-}$mice and octanoyl ghrelin levels were not affected after RYGB surgery. In WT mice, nutrients act via α-gustducin to increase L-cell differentiation (foregut) and L-cell number (foregut and hindgut) in a region-dependent manner. In *α-gust*$^{-/-}$mice, the effect on gut hormone levels is probably tuned via increased peptide sensor and glucose transporter expression in the Roux limb and increased caecal butyrate and propionate levels in the hindgut that activate FFARs. Finally, signaling via α-gustducin plays a role in the increased ion transport of the foregut but not in the improvement in colonic barrier function.

In conclusion, RYGB surgery decreased body weight in both WT and *α-gust*$^{-/-}$mice. Elevated plasma GLP-1 and PYY levels might mediate this effect, although α-gustducin differentially affects several regulatory systems in the foregut and hindgut, tuning gut hormone release.

5.2 Introduction

The obesity epidemic is posing a major healthcare problem worldwide. Roux-en-Y gastric bypass (RYGB) surgery induces sustained weight loss and remission of comorbidities (Schauer et al., 2012). Altered gut hormone release is considered as one of the possible mechanisms for the post-surgical metabolic improvements (Svane et al., 2015). RYGB surgery enhances the secretion of the anorexigenic hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), and although more controversial, inhibits secretion of the orexigenic hormone ghrelin (Sweeney and Morton, 2014). The mechanisms involved are incompletely understood.

After RYGB surgery, the contact of nutrients with much of the stomach and duodenum is bypassed, resulting in a rapid delivery of undigested nutrients to the jejunum. This rerouting could affect the nutrient sensing mechanisms controlling gut hormone release.

EECs in the gut sense nutrients through taste receptors (TASRs) and chemosensory pathways similar to those on the tongue to regulate meal-induced gut hormone secretion (Depoortere, 2014). The sweet TASR (TAS1R2-TAS1R3) and sodium-dependent glucose co-transporter 1 (SGLT1) function as glucose-sensors of the L-cell (Jang et al., 2007, Gorboulev et al., 2011). Amino acid sensing is tuned by the umami TASR (TAS1R1-TAS1R3), the metabotropic glutamate receptors, the calcium sensing receptor, G protein-coupled receptor class C group 6 member A and lysophosphatidic acid receptor 5 (LPAR5) (Wellendorph and Brauner-Osborne, 2009). Subtypes of FFAR sense short-chain fatty acids (SCFAs) (FFAR2, FFAR3) and medium- and long chain fatty acids (FFAR1, FFAR4) on EECs (Hara et al., 2014).
α-*gust*ducin, the α-subunit of the G-protein coupled to TASRs, plays an important role in taste transduction and is colocalized with several, but not all, TASRs on EECs (McLaughlin et al., 1992, Janssen et al., 2011, Jang et al., 2007). For example, *glucose- and SCFA induced* GLP-1 release and bitter-induced ghrelin release is blunted in α-*gust*ducin knockout (α-*gust*$^{-/-}$) mice (Jang et al., 2007, Li et al., 2013, Janssen et al., 2011).
Additionally, nutrient rerouting after RYGB surgery modifies the gut microbiome (Furet et al., 2010) resulting in altered microbial fermentation products such as SCFAs (acetate, butyrate, propionate) which may regulate energy -and glucose homeostasis via FFAR2 and FFAR3 signaling on EECs (Canfora et al., 2015).
Butyrate can also enhance the impaired intestinal barrier function, associated with obesity, by facilitating tight junction assembly (Moreno-Navarrete et al., 2012, Peng et al., 2009).

The present study investigated the role of the nutrient sensing pathway in the metabolic reprogramming associated with RYGB surgery. We hypothesized that the new digestive route after RYGB surgery affects the gustducin-mediated taste receptor signaling pathway that partially controls gut hormone secretion to regulate body weight and glucose homeostasis. Furthermore we elucidated whether gustducin-mediated signaling plays a role in the morphological changes in the mucosa, the enteroplasticity of EECs and the restoration of the 'leaky gut' after RYGB surgery. To test these hypotheses, we compared body weight, glucose tolerance, mucosal thickness, nutrient sensor expression, SCFA production, gut hormone release/expression and gut permeability/transepithelial resistance in diet-induced obese wild type (WT) and *α-gust*$^{-/-}$*mice*, 7 weeks after RYGB surgery.

5.3 Material and methods

5.3.1 Animals

Male C57BL/6 WT and *α-gust*$^{-/-}$mice (Dr. R. Margolskee, Monell Chemical Senses Center, Philadelphia, USA) were kept in the animal facility (20–22°C) under a 14-h:10-h light-dark cycle and had ad libitum access to food and drinking water. All experimental procedures were approved by the Ethical committee for Animal Experiments of the KU Leuven (P100-2013).

5.3.2 Experimental design

6 week-old WT and *α-gust*$^{-/-}$mice were fed a western style diet (TD.08811, 45% kcal fat 41% kcal carbohydrate, Harlan Laboratories Inc.) for 12 weeks. Mice were randomized in three groups: a sham-group fed ad libitum (ALF), a sham-group, pair-fed to the RYGB group of its respective genotype (PF), and a RYGB group fed ad libitum (RYGB) (see Supplementary material). All mice received the western style diet for 7 weeks till sacrifice (Fig. S1).

5.3.3 Post-operative analyses

Post-surgical body weight and food intake were monitored. All pair-fed mice received the same amount of food per day as their respective RYGB group ate on that postsurgical day. Seven weeks post-surgery, fasted (6h) mice were gavaged with Nutridrink® (Nutricia) 15 minutes before sacrifice. Blood was collected by cardiac puncture and supplemented with AEBSF (4mM) and EDTA (1mg/ml). Plasma was supplemented with dipeptidyl peptidase 4 inhibitor (10µl/ml) (for GLP-1 and PYY) or acidified (for ghrelin). Tissue segments were sampled as illustrated (Fig. 3A) and stored in paraformaldehyde (4%) or RNA later at -80°C for further analysis. The distal colon and common limb, or corresponding segment, were used for permeability experiments. See Supplementary material for a detailed description of the post-operative analyses.

5.3.4 Statistical analyses

All values are expressed as mean ± SEM. Changes in body weight, food intake, glucose tolerance, plasma insulin levels, TEER and fluorescein levels over time between different genotypes and operations were analyzed using a repeated measures mixed models analysis (SAS software package 9). Other data were analyzed with a two-way ANOVA, followed by planned comparisons post-hoc testing, corrected for multiple testing with Bonferroni-Holm correction (Statistica 12, Statsoft). Significance was accepted at the 5% level.

5.4 Results

5.4.1 RYGB induced body weight loss in WT and *α-gust*$^{-/-}$ mice

Postsurgical body weight, food intake, fat mass and plasma leptin levels were measured in WT and *α-gust*$^{-/-}$ mice.

Body weight did not differ between the different genotypes and groups before surgery or at the time of surgery (Fig. 1A, B, S2A). Body weight decreased in the first week after surgery in all groups to a similar extent (Fig. 1A, B). Body weight completely recovered in WT ALF mice but not in *α-gust*$^{-/-}$ ALF mice (-15%, $P<0.05$) (Fig. 1A, B). The latter group also showed lower plasma leptin levels (-46%, $P<0.05$) (Fig. 1D) at sacrifice.

Pair-feeding (to determine the effects due to caloric restriction) and RYGB surgery decreased body weight in a more pronounced manner in WT mice ($operation_{ALF\text{-}PF}$*genotype*time; $P<0.001$) ($operation_{ALF\text{-}RYGB}$*genotype*time; $P<0.05$) compared to *α-gust*$^{-/-}$ mice (Fig. 1A, B). This interaction effect may be partially due to the genotype-dependent effect of sham surgery on body weight ($operation_{ALF}$*genotype*time; $P<0.05$). Therefore we cannot draw any important conclusions about the role of α-gustducin in the effect of pair-feeding or RYGB surgery on body weight loss. Postoperative cumulative food intake was decreased ($P<0.05$) in WT RYGB but not in *α-gust*$^{-/-}$ RYGB mice compared to ALF mice (Fig. 1C).

However, PF mice weighed more than RYGB mice in week 1-7 ($operation_{PF\text{-}RYGB}$*time; WT: $P<0.001$, *α-gust*$^{-/-}$: $P<0.01$) in both genotypes, indicating that diminished food intake is only partially responsible for the body weight loss after RYGB surgery (Fig. 1A, B). Total fat pad mass ($P<0.001$) and plasma leptin levels (WT: $P<0.001$, *α-gust*$^{-/-}$: $P<0.01$) were reduced after RYGB surgery in both genotypes (Fig. 1D, E).

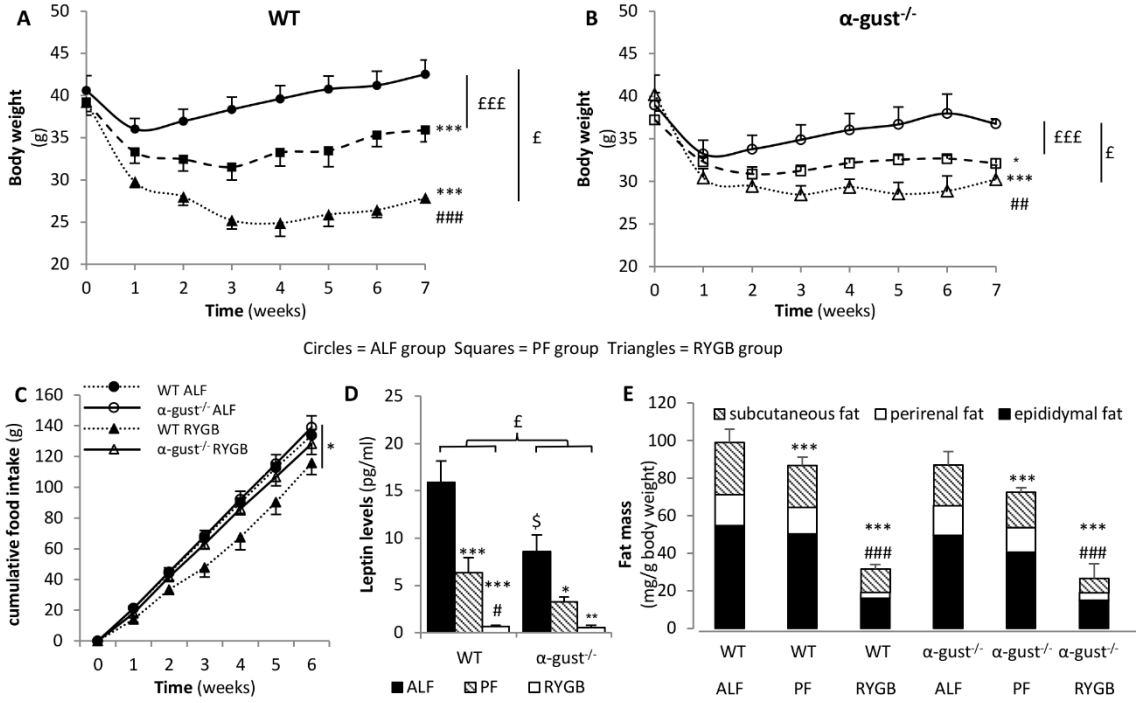

Figure 1: RYGB surgery induced weight loss in WT and *α-gust*$^{-/-}$mice. (**A, B**) Time-dependent changes in body weight in ALF, PF and RYGB groups in WT and *α-gust*$^{-/-}$mice (n= 7-9). (**C**) Cumulative food intake (7 weeks) in ALF and RYGB groups in WT and *α-gust*$^{-/-}$mice (n= 5-9). (**D**) Plasma leptin levels in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 5-9). (**E**) Total fat mass of ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 6-9). *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001 vs. ALF groups. #: *P*<0.05, ###: *P*<0.001 vs. PF groups. $ *P*<0.05 vs. WT ALF mice. £ *P*<0.05, £££ *P*<0.001 genotype*operation effect.

5.4.2 RYGB improves glucose homeostasis in WT mice while *α-gust*$^{-/-}$mice are protected from the diabetogenic effect of a western diet

An oral glucose tolerance test was performed and serum insulin levels were determined to elucidate the effect of RYGB surgery on glucose homeostasis. Fasting blood glucose and serum insulin levels were used to measure the insulin resistance by calculating the insulin resistance index.

Two weeks before surgery and five weeks after sham-surgery *α-gust*$^{-/-}$mice were less glucose intolerant than WT mice (*P*<0.05) (Fig. 2A, B, S2B). Their serum insulin profiles did not statistically differ (Fig. 2C, D). Accordingly, insulin resistance was lower (*P*<0.05) in *α-gust*$^{-/-}$ ALF mice compared to WT ALF mice (Fig. 2E).

RYGB surgery and pair-feeding improved glucose tolerance (*P*<0.05) in WT but not in *α-gust*$^{-/-}$ mice (Fig. 2A, B). However, RYGB surgery resulted in a fast glucose response after an oral

glucose challenge, while pair-feeding was associated with lower blood glucose levels at all time points (Fig. 2A).

WT PF (P<0.01) and WT RYGB mice (P<0.05) showed lower plasma insulin levels during the oral glucose tolerance test (Fig. 2C), suggesting that the improvement in glucose tolerance was due to changes in insulin resistance. Indeed, the insulin resistance decreased in WT PF and WT RYGB mice (P<0.001) to a similar extent (Fig. 2E).

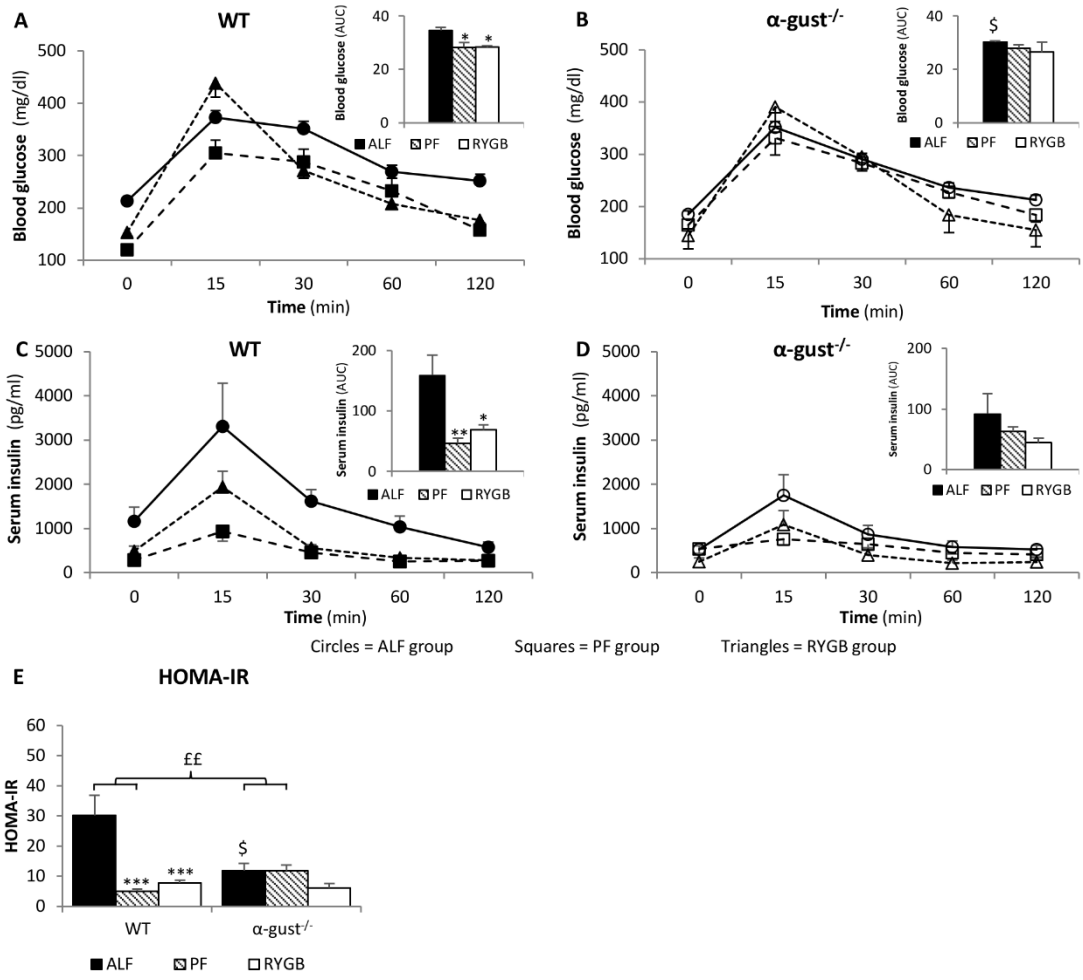

Figure 2: RYGB improves glucose homeostasis and insulin resistance in WT mice. (**A, B**) plasma glucose and (**C, D**) serum insulin levels during an oral glucose tolerance test (OGTT) (2g/kg) in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n=5-8). (**E**) Insulin resistance index (HOMA-IR) of ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 5-8). * P<0.05, ** P<0.01, *** P<0.001 vs. ALF groups. $ P<0.05 vs. WT ALF mice. ££ P<0.01 genotype*operation effect.

5.4.3 α-gustducin plays a role in the morphological changes induced by nutrient rerouting

Bariatric surgery can lead to structural changes in gut morphology, especially in regions overexposed to nutrients (le Roux et al., 2010, Taqi et al., 2010). This raises the possibility that nutrient sensing mechanisms may directly regulate these effects.

Fig. 3A represents a schematic illustration of the postoperative anatomy after RYGB surgery and the sampled corresponding segments in the sham-groups. A representative hematoxylin and eosin stained section of the Roux limb (RL) (RYGB group) and corresponding jejunum (sham-group) of both genotypes is illustrated in Fig. 3B. RYGB decreased (P<0.05) the mucosal height of the biliopancreatic limb (BPL) of WT, but not of *α-gust*$^{-/-}$mice, compared to the duodenum of ALF mice (Fig. 3C, D). The mucosal height of the RL was increased in a genotype-dependent manner compared to the jejunal segment of ALF mice ($operation_{ALF\text{-}RYGB}$*genotype; P<0.05) (Fig. 3C, D). No changes were observed in the common limb (CL). These morphological changes did not occur after pair-feeding (data not shown). GLP-2 receptor mRNA levels in the RL were increased (P<0.05) in both genotypes, despite the absence of mucosal hypertrophy in *α-gust*$^{-/-}$*mice* (Fig. 3E, F).

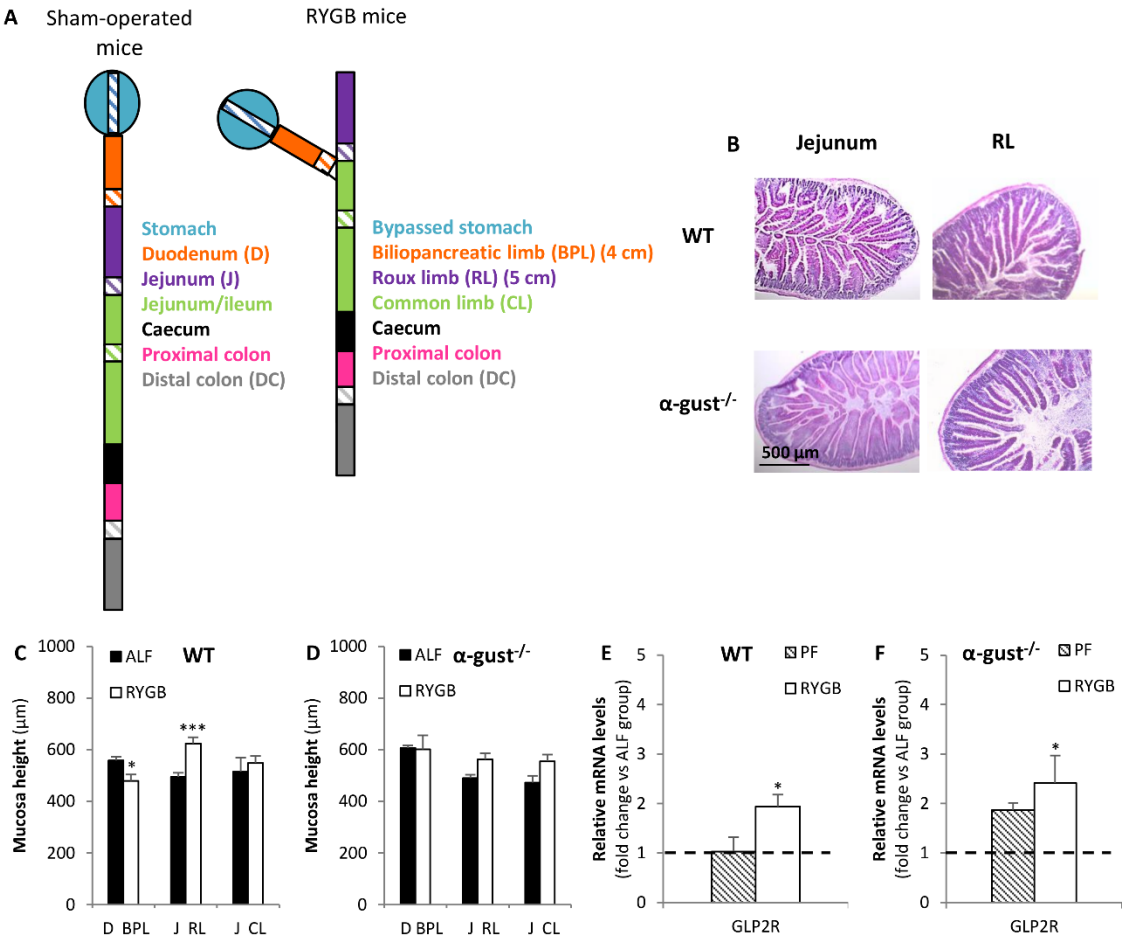

Figure 3: RYGB induces changes in gut morphology in an α-gustducin-dependent manner. (**A**) Schematic illustration of the postoperative anatomy after RYGB surgery and the sampled corresponding segments in the sham-operated groups. (**B**) Representative Hematoxylin & Eosin (H&E) staining of the RL (RYGB group) and the jejunum (ALF group) of both genotypes. (**C, D**) mucosal height from sections from the limbs (BPL, RL, CL) or corresponding segments of sham-groups (duodenum, jejunum) of WT and *α-gust*$^{-/-}$mice (n= 5). (**E, F**) Relative GPL2R mRNA expression in the RL (RYGB) or jejunum (ALF, PF) in WT and *α-gust*$^{-/-}$mice (n= 6-9). The dotted line indicates the mean mRNA levels in ALF mice. * P<0.05, *** P<0.001 vs. ALF groups.

5.4.4 The effect of nutrient rerouting on nutrient sensor expression differs between WT and *α-gust*$^{-/-}$mice

We hypothesize that the absence of nutrients in the BPL or the exposure to undigested nutrients in the RL after RYGB surgery may alter nutrient sensor expression and signaling on EECs resulting in altered gut hormone release.

The mRNA expression of the following nutrient sensors was determined in the RL and BPL: a) the sweet taste receptors (TAS1R2-TAS1R3) and consequently one of the subunits of the umami taste receptor (TAS1R3), b) the glucose transporters SGLT1 and glucose transporter 2 (GLUT2), c) the di-tri peptide sensor LPAR5 and d) the medium/long chain fatty acid sensor FFAR4.

Pair-feeding did not affect mRNA levels of nutrient sensors. In WT mice, RYGB surgery increased (P<0.001) LPAR5 mRNA levels in the BPL but did not affect nutrient sensor expression in the RL (Fig. 4A).

In *α-gust*$^{-/-}$mice, RYGB surgery decreased (P<0.01) TAS1R3 mRNA levels compared to the PF group in the BPL, while it increased GLUT2 ($operation_{ALF\text{-}RYGB}$*genotype; P<0.05) and LPAR5 (P<0.05) mRNA levels in the RL (Fig. 4B).

5.4.5 SCFA production and the nutrient sensing mechanisms in the distal gut differ between WT and *α-gust*$^{-/-}$mice

The nutrient rerouting after RYGB surgery will also affect bacterial fermentation in the distal gut. RYGB surgery increased wet caecal weight (WT; +63±23g, *α-gust*$^{-/-}$; +46±18g) compared to ALF groups. Furthermore, caecal butyrate and propionate levels were increased (P<0.001) in *α-gust*$^{-/-}$mice, but not in WT mice ($operation_{ALF\text{-}RYGB}$*genotype; P<0.001) (Fig. 4C-E). These alterations were not the result of an altered caloric intake (PF group).

The increased SCFA production in *α-gust*$^{-/-}$mice, was accompanied by decreased colonic FFAR2 mRNA levels (P<0.01). Furthermore, RYGB surgery decreased colonic FFAR3 mRNA levels in both genotypes (P<0.001) (Fig. 4A, B).

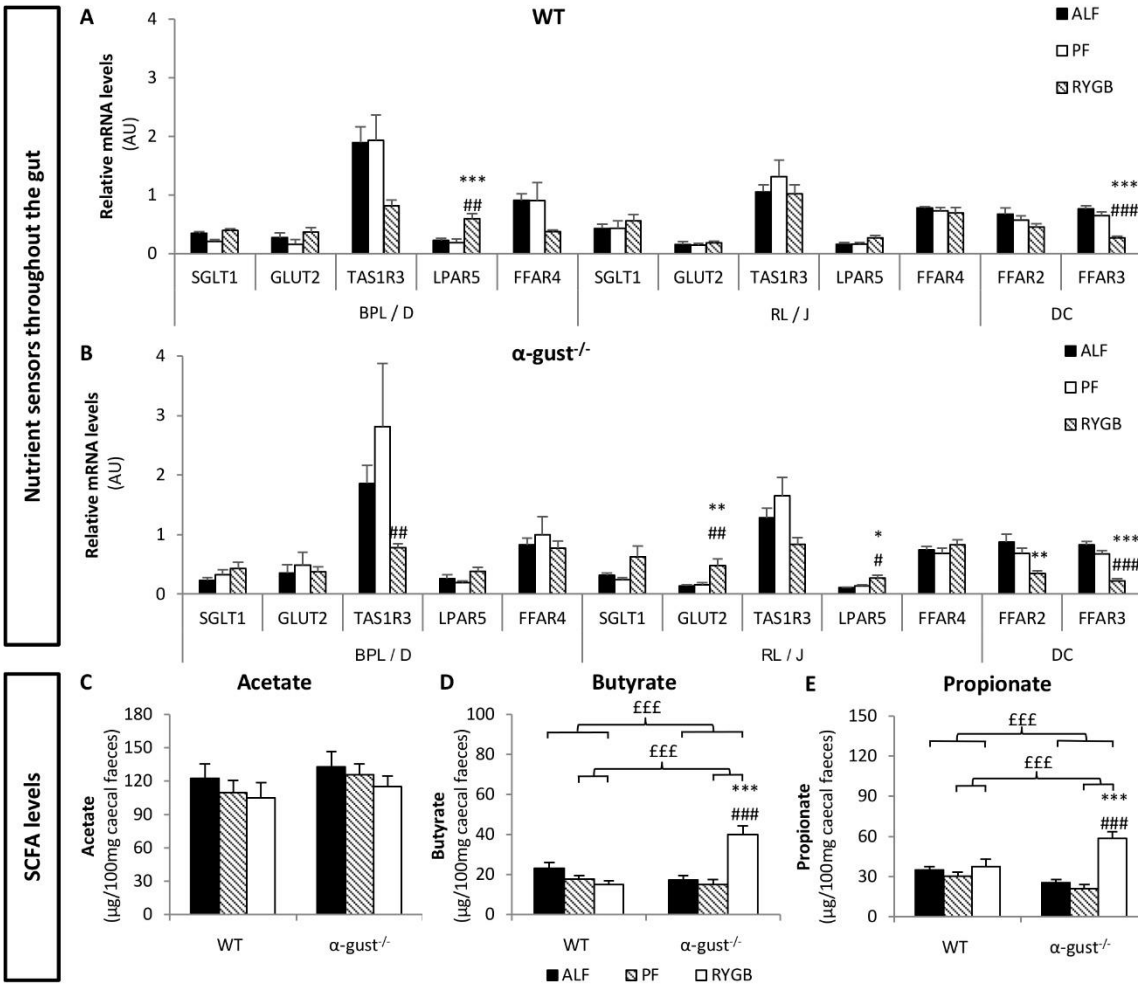

Figure 4: Effect of RYGB on different elements of the nutrient sensing machinery. (**A, B**) Relative mRNA levels of the monosaccharide transporters (SGLT1, GLUT2), the sweet-and umami taste receptor subunit (TAS1R3), the peptide sensor (LPAR5), the fatty acid sensor (FFAR4) and the SCFA receptors (FFAR2, FFAR3) in the BPL, RL or colon (RYGB) and jejunum, duodenum or colon (ALF, PF) in WT and α-gust-/- mice (n= 7-9). (C-E) Caecal SCFA levels 15 min after nutridrink gavage in ALF, PF and RYGB WT and α-gust-/-mice (n = 6-9). Results are presented as mean ± SEM. AU: arbitrary units. * *P*<0.05, ** *P*<0.01, *** *P*<0.001, vs. ALF groups. # *P*<0.05, ## *P*<0.01, ### *P*<0.001, vs. PF groups. £££ *P*<0.001 genotype*operation effect.

5.4.6 α-gustducin plays a role in the increased postsurgical plasma PYY levels, but not in the increased GLP-1 levels

The altered gut morphology and nutrient sensor mRNA expression may affect the release of gut hormones, key regulators of the energy -and glucose homeostasis. Plasma ghrelin, GLP-1 and PYY levels were determined. Immunohistochemical studies and real-time PCR was performed in the different limbs to determine the origin of these hormonal changes.

5.4.6.1 Ghrelin levels

RYGB surgery did not alter meal-induced (15 min) plasma octanoyl ghrelin levels but increased (P<0.01) total (octanoyl+desoctanoyl) ghrelin levels in *α-gust*$^{-/-}$mice, but not in WT mice (Fig. 5A, B). Pair-feeding did not affect plasma ghrelin levels.

The number of octanoyl or total ghrelin immunoreactive cells in the bypassed stomach, RL or BPL was not affected in either genotype (Fig. S3A-F). A representative immunofluorescence co-staining between gastric octanoyl and total ghrelin immunoreactive cells is shown in Fig. 5C.

The increased plasma total ghrelin levels in *α-gust*$^{-/-}$mice were accompanied with decreased ghrelin mRNA levels in the BPL (P<0.01) and RL (P<0.05), but not in the bypassed stomach, the main production site of ghrelin. Ghrelin mRNA levels were decreased in WT mice in the BPL after pair-feeding (P<0.01) and RYGB surgery (P<0.001), but this did not result in altered plasma ghrelin profiles (Fig. 5D, E). The mRNA levels of ghrelin-O-acyl transferase, the enzyme catalyzing the octanoylation of ghrelin, were not altered in the bypassed stomach or different limbs of either genotype (Fig. S3J, K).

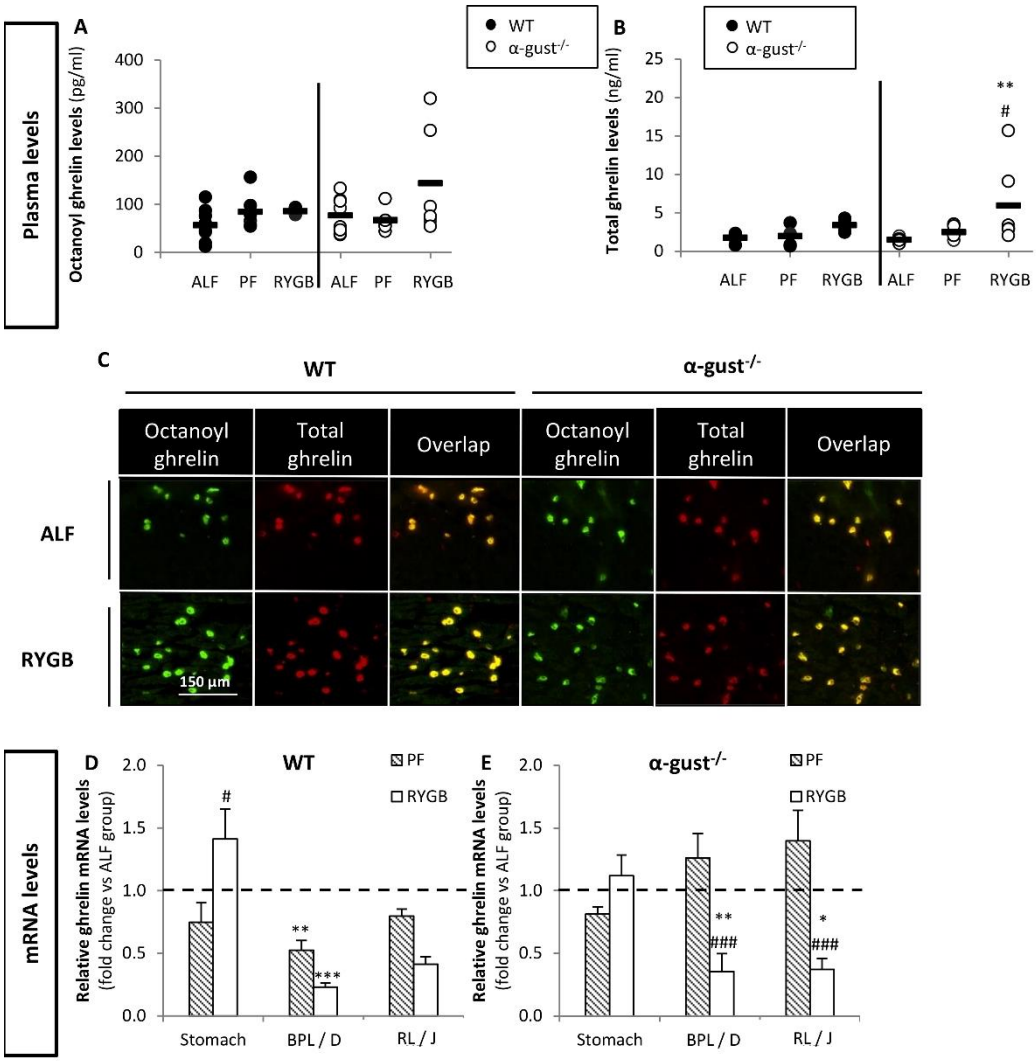

Figure 5: RYGB increased plasma total ghrelin levels in *α-gust*$^{-/-}$mice. (**A, B**) Meal-induced (15 min) plasma octanoyl and total ghrelin levels in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 5-9). (**C**) Double-immunofluorescence staining for octanoyl and total ghrelin in stomach sections of ALF and RYGB mice. (**D, E**) Relative ghrelin mRNA expression in the bypassed stomach, BPL or RL (RYGB) or corresponding small intestine (ALF, PF) in WT and *α-gust*$^{-/-}$mice (n= 5-8). The dotted line indicates the mean mRNA levels in ALF mice. *: *P*<0.05, ***: *P*<0.001 vs. ALF groups. #: *P*<0.05, vs. PF groups. £ *P*<0.05, ££ *P*<0.01.

5.4.6.2 GLP-1 levels

RYGB surgery, but not pair-feeding, increased (P<0.001) meal-induced plasma GLP-1 secretion in both genotypes (Fig. 6A).

GLP-1 levels may originate from L-cells in the proximal or distal gut. In the BPL, no changes in the number of GLP-1 immunoreactive cells were observed in either genotype (Fig. 6B). In the RL, the genotype-dependent increase in mucosal thickness was accompanied by an increase (P<0.001) in the number of GLP-1 immunoreactive L-cells in WT mice (Fig. 6C), but not in *α-gust*$^{-/-}$mice (operation$_{ALF\text{-}RYGB}$*genotype; P<0.01). Furthermore L-cell density, obtained after normalization for the section area, was increased with 89% in WT but not in *α-gust*$^{-/-}$mice (operation$_{ALF\text{-}RYGB}$*genotype; P<0.01) and was accompanied by a downregulation (P<0.05) of the entero-endocrine differentiation marker neurogenin 3 (Ngn3) (Fig. S4B-D) (Gradwohl *et al.* 2000). Proglucagon mRNA levels in the RL and BPL were not affected (Fig. 6E, F).

Additionally, RYGB surgery induced a genotype-dependent increase in the number of colonic GLP-1 immunoreactive cells of WT but not of *α-gust*$^{-/-}$mice (Fig. 6D). This was probably due to a thicker mucosa of the distal colon which tended (P=0.09) to increase after RYGB surgery (Fig. S4A), resulting in an unchanged L-cell density (Fig. S4E). Accordingly colonic Ngn3 mRNA levels were not altered by RYGB surgery or pair-feeding in both genotypes (Fig. S4F, G). RYGB surgery decreased (P<0.001) colonic proglucagon mRNA transcripts of *α-gust*$^{-/-}$mice, but not of WT mice (Fig. 6E, F).

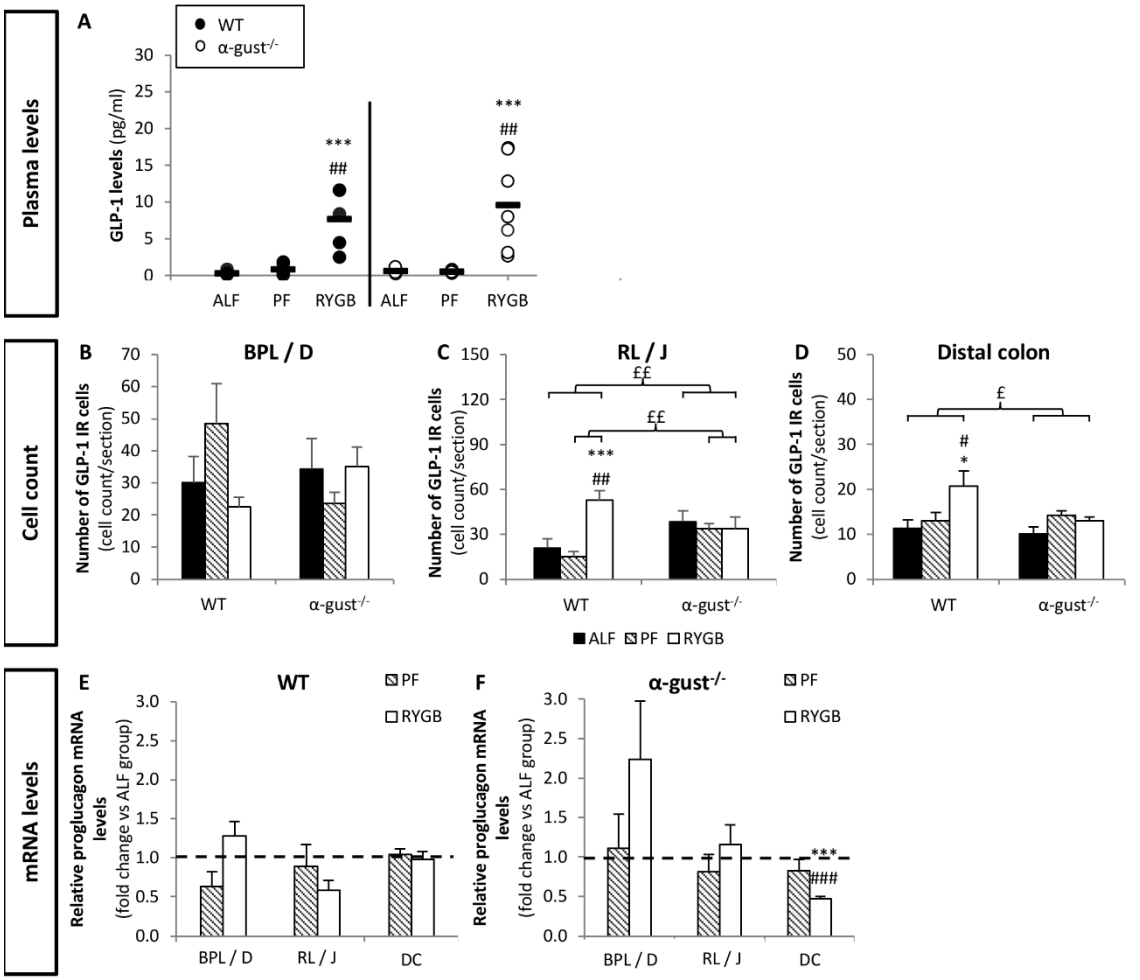

Figure 6: RYGB increased plasma GLP-1 levels in an α-gustducin-independent manner. (**A**) Meal-induced (15 min) plasma levels of GLP-1 (7-36)amide and GLP-1 (7-37) in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 6-9). Number of GLP-1 immunoreactive cells in sections of the BPL (**B**), RL (**C**) and distal colon (**D**) in RYGB groups or corresponding segment (ALF, PF) in WT and *α-gust*$^{-/-}$mice (n = 5). (**E, F**) Relative proglucagon mRNA expression in the BPL, RL, DC (RYGB) or corresponding segment (duodenum, jejunum, distal colon (ALF-PF) in WT and *α-gust*$^{-/-}$mice (n= 5-8). The dotted line indicates the mean mRNA levels in ALF mice. *: *P*<0.05, ***: *P*<0.001 vs. ALF groups. #: *P*<0.05, ##: *P*<0.01 vs. PF groups. £ *P*<0.05, ££ *P*<0.01 genotype*operation effect: £ *P*<0.05, ££ *P*<0.01.

5.4.6.3 PYY levels

RYGB surgery, but not pair-feeding, significantly increased postprandial plasma PYY levels in *α-gust*$^{-/-}$mice, and tended (*P*=0.07) to increase plasma PYY levels in WT mice resulting in an interaction effect (operation$_{ALF\text{-}RYGB}$*genotype; *P*<0.001) (Fig. 7A).

RYGB surgery increased the number (*P*<0.05), but not the density, of colonic PYY immunoreactive L-cells in WT but not in *α-gust*$^{-/-}$mice (operation$_{ALF\text{-}RYGB}$*genotype; *P*<0.05) (Fig. 7B). A representative immunostaining showing colocalization between GLP-1 and PYY

containing L-cells in the colon is shown in Fig. 7E. Under ALF conditions 27% and 20% of the L-cells only stained for GLP-1 or PYY respectively. After RYGB surgery, no shift in any subpopulation was observed (30% GLP-1, 16% PYY). Furthermore RYGB surgery increased (*P*<0.001) colonic PYY mRNA levels in both genotypes (Fig. 7C, D).

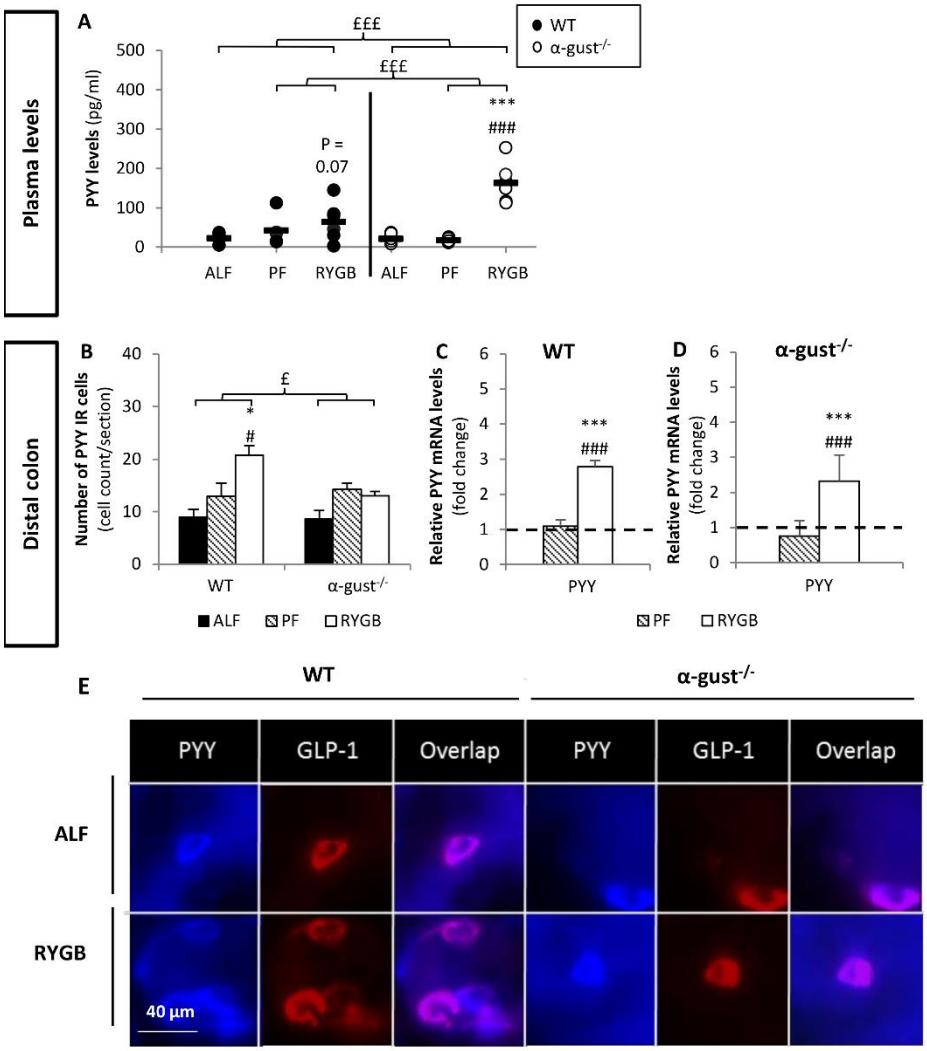

Figure 7: RYGB increased plasma PYY levels in an α-gustducin-dependent manner. (**A**) Meal-induced (15 min) plasma levels of PYY(1-36) and PYY(3-36) in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 6-9). (**B**) Number of PYY immunoreactive cells in the distal colon in ALF, PF and RYGB WT and *α-gust*$^{-/-}$mice (n = 5). (**C, D**) Relative PYY mRNA expression in the distal colon in PF and RYGB WT and *α-gust*$^{-/-}$mice (n= 5-8). The dotted line indicates the mean mRNA levels in ALF mice. (**E**) Double-immunofluorescence staining for GLP-1 and PYY in sections from the distal colon of ALF and RYGB WT and *α-gust*$^{-/-}$mice. *: *P*<0.05, ***: *P*<0.001 vs. ALF groups, #: *P*<0.05, ###: *P*<0.001 vs. PF groups. £ *P*<0.05, £££ *P*<0.001 genotype*operation effect: £ *P*<0.05, £££ *P*<0.001.

5.4.7 The RYGB-induced alterations in ion secretion in the foregut are α-gustducin-dependent, while the altered gut permeability is α-gustducin-independent

Via alterations in intestinal permeability, intestinal barrier function becomes compromised during obesity whereby access of dietary antigens to mucosal immune elements is facilitated. We investigated whether RYGB can restore the 'leaky gut' associated with obesity.

Epithelial integrity of tissue segments was evaluated in Ussing chambers by measuring trans-epithelial electrical resistance (TEER) and fluorescein passage (permeability). Ion transport was calculated (short circuit current; Isc).

RYGB surgery, but not pair-feeding, increased the Isc of the CL in WT mice ($P<0.001$) but not in *α-gust*$^{-/-}$mice, compared to the corresponding segment in the sham-operated group ($operation_{ALF\text{-}RYGB}$*genotype; $P<0.01$). Colonic Isc was unaffected (Fig. S5A-F).

RYGB surgery and pair-feeding did not affect TEER in the CL (data not shown) or colon (Fig. S6A, B) in both genotypes. However, RYGB mice showed a genotype-independent decrease (WT; $P<0.05$, *α-gust*$^{-/-}$; $P<0.01$) in colonic fluorescein passage compared to ALF mice (Fig. S6C, D), while fluorescein passage of the CL was unaffected (data not shown). Colonic mRNA levels of the tight junction protein occludin (Fig. S6E, F), which is important in the leak pathway regulation, were decreased in both genotypes after RYGB surgery (Buschmann et al., 2013).

5.5 Discussion

Bariatric surgery is not just an effective treatment option for obesity, but a platform that can yield new insights into the etiology of metabolic diseases.

The idea behind the 'restrictive' RYGB surgery was that creation of a small pouch would reduce the amount of calories that would be consumed. In agreement with other studies, our results indicate that a decreased post-surgical food intake cannot fully explain the reduction in body weight since pair-feeding resulted in a less pronounced weight loss compared to RYGB surgery (Mokadem et al., 2014, Reddy et al., 2014). Increased EE as reported in mice and rats after RYGB surgery, is likely to contribute to the additional body weight loss (Nestoridi et al., 2012, Bueter et al., 2010).

In the current study, α-gust$^{-/-}$mice responded differently to the sham operation, resulting in a lower body weight at sacrifice. This genotype-dependent effect may play a role in the effect of RYGB surgery on the body weight loss in these mice. Therefore we could not clearly assess the role of α-gustducin in the RYGB-induced body weight loss. A previous study also reported comparable RYGB-induced body weight loss between WT and *α-gust*$^{-/-}$mice (Mokadem et al., 2014).

Solely a reduction in food intake seems to be sufficient to improve glucose homeostasis after gastric bypass since the WT pair-fed group also showed an improved glucose tolerance and insulin resistance.
This observation has been made previously after an intra-peritoneal glucose tolerance test in mice that underwent RYGB, vertical sleeve gastrectomy (VSG) or pair-feeding (Chambers et al., 2011). In contrast, an oral glucose tolerance test in obese insulin-resistant Zucker rats showed that RYGB, but not pair-feeding improved glucose homeostasis (Meirelles et al., 2009). Results in humans also suggest that caloric restriction underlies the short-term metabolic benefits of RYGB since a very low caloric diet and RYGB showed similar effects on glucose homeostasis (Lips et al., 2014).

The role of α-gustducin in the RYGB-induced improvement of glucose homeostasis could not be clearly assessed since sham-operated *α-gust*$^{-/-}$mice displayed better glucose profiles and tended to display lower insulin levels compared to sham-operated WT mice. These results indicate that *α-gust*$^{-/-}$mice were partially protected from the diabetogenic properties of a western style diet. Avau *et al.* showed that high-fat diet-induced obese *α-gust*$^{-/-}$mice have an increased heat production compared to WT mice, as a result of an increased brown adipose tissue thermogenic activity (Avau et al., 2015), this could explain why sham operated *α-gust*$^{-/-}$ mice did not completely regain weight after surgery and were less glucose intolerant.

The increased exposure of the gut to undigested nutrients after RYGB surgery can result in structural changes in gut morphology (Seeley et al., 2015). Our study provides novel mechanistic insights demonstrating that *α-gust*$^{-/-}$mice do not 'sense' the need for morphological adaptations when deprived (BPL) or overexposed (RL) to undigested nutrients after RYGB. Increased plasma GLP-2 levels (trophic hormone) in rats and humans have been shown to correlate with increased crypth cell proliferation after RYGB surgery (le Roux et al., 2010, Seeley et al., 2015). In our study, GLP-2 receptor mRNA levels increased in the RL of both WT and *α-gust*$^{-/-}$mice, in contrast to the increased mucosal thickness which was genotype-dependent. These findings suggest that the increased mucosal thickness is not GLP-2 receptor mediated or that α-gustducin plays a role in the GLP-2 receptor transduction cascade.
Previous studies showed that alterations in gut morphology increased the number of L-cells in the RL (Mumphrey, 2013). We additionally highlighted similar changes in the distal gut and showed that L-cell number and/or density was affected in WT but not in *α-gust*$^{-/-}$mice. Obesity per se is not the trigger for these changes in WT mice. Indeed, obese *ob/ob* mice did not show altered L-cell density while mice on a high fat diet did, highlighting the role of nutrient sensing and a nutrient rich environment in the changes in L-cell count (Aranias et al., 2015).

The increased L-cell density in the RL suggests that the increased L-cell number is not solely due to the morphological changes. Additionally, the α-gustducin-dependent compensatory decrease of the differentiation marker Ngn3 may indicate that sensing the luminal content through gustducin triggers the differentiation of progenitor cells towards EECs instead of enterocytes (Gradwohl et al., 2000).

If the nutrient sensing cascade is disrupted (*α-gust*$^{-/-}$mice), the gut may compensate by upregulating nutrient sensors on EECs to regulate gut hormone secretion. Indeed, *α-gust*$^{-/-}$ mice, but not WT mice, showed increased LPAR5 and GLUT2 mRNA levels in the RL. Similarly, in a RYGB rat model mRNA expression levels of TAS1R2, SGLT1 and GLUT2 remained unaltered in the RL of WT mice (Bhutta et al., 2014). Nevertheless, a human study reported an upregulation of SGLT1 and GLUT2 but no change in sweet TASR expression in the RL after RYGB surgery (Nguyen et al., 2014). The sweet TASR has been shown to regulate the expression of SGLT1 and GLUT2 via a sweet taste receptor or α-gustducin-dependent signaling process (Margolskee et al., 2007, Mace et al., 2009). Therefore the lack of α-gustducin-mediated signaling could lead to a compensatory upregulation of GLUT2.
One of the limitations of this study is that the protein levels of the different nutrient receptors were not investigated.

The nutrient rerouting after RYGB surgery can also modify the gut microbiome (Furet et al., 2010) resulting in an altered bacterial fermentation. The SCFAs acetate, butyrate and propionate are important microbial fermentation products. Literature concerning the effect of bariatric surgery on SCFA production is limited (Liou et al., 2013, Tremaroli et al., 2015). Caecal butyrate and propionate but not acetate levels were increased in *α-gust*$^{-/-}$mice but not in WT mice, suggesting that α-gustducin mediated sensing mechanisms may be linked to bacterial fermentation. A previous rodent study showed increased ceacal propionate levels but decreased acetate levels after RYGB surgery (Liou et al., 2013). This discrepancy may be related to differences in the diet (high-fat vs western style) and period of fasting (2h vs 6h) (Liou et al., 2013). In humans, RYGB surgery tended to decrease fecal SCFA levels 10 years after RYGB surgery (Tremaroli et al., 2015).
The increased SCFA levels in *α-gust*$^{-/-}$mice may trigger gut hormone release. *In vitro* and *in vivo* studies showed that SCFAs stimulate the secretion of GLP-1 and PYY from L-cells in a FFAR2, and FFAR3 dependent manner (Tolhurst et al., 2012). However, the selective increase of propionate and butyrate in *α-gust*$^{-/-}$mice did not correlate with the genotype-independent downregulation of FFAR3, but it did correlate with the FFAR2 mRNA transcripts, which were

selectively decreased in *α-gust*$^{-/-}$mice. These mRNA levels might reflect a compensatory downregulation due to increased SCFA exposure.
Diet-induced obese FFAR2$^{-/-}$mice have a higher EE, higher core body temperature and decreased adiposity (Bjursell et al., 2011) suggesting that SCFAs can regulate EE via FFAR2 signaling. Therefore the increase in SCFA production in *α-gust*$^{-/-}$mice might lower EE through FFAR2 signaling, explaining the decrease in body weight after RYGB surgery.

Butyrate can also improve intestinal permeability by decreasing paracellular passage through the facilitation of tight junction assembly (Peng et al., 2009, Cani et al., 2008). However, the genotype-dependent increase in butyrate levels cannot explain the genotype-independent nature of the decreased colonic paracellular passage of large molecules. Consequently, the altered expression of the tight junction protein occludin in both genotypes may explain the improvement in colonic leak passage but will not be due to altered butyrate levels.
Furthermore, RYGB did not affect mucosal integrity in the proximal intestine, in contrast to a previous report which showed improvement in human proximal small intestine permeability six to eight months after RYGB surgery (Casselbrant et al., 2015).
The morphological changes in the proximal gut in combination with the changes in nutrient sensor expression and altered bacterial fermentation in the distal gut after RYGB surgery may alter gut hormone profiles (Seeley et al., 2015, Umeda et al., 2011).

In our study plasma octanoyl ghrelin levels were not significantly altered, although total ghrelin levels were selectively increased in *α-gust*$^{-/-}$mice. Since the octanoylated form of ghrelin is biologically active, it is unlikely that plasma ghrelin levels contribute to the weight loss after RYGB. This is consistent with published data where vertical sleeve gastrectomy induced comparable effects on food intake and body weight in wild type and ghrelin-deficient mice (Chambers et al., 2013). Nevertheless, the decreased ghrelin mRNA levels in the BPL and RL indicate that nutrient rerouting enables the limbs, in addition to the stomach, to play a role in the regulation of plasma ghrelin secretion. The importance of nutrient sensing via TASRs in peptone-induced ghrelin secretion was already shown in a ghrelinoma cell line and in jejunal segments (Vancleef et al., 2015).

The increase of postprandial GLP-1 levels after RYGB surgery has consistently been reported in rodents and humans (Miras and le Roux, 2013). Changes in the foregut and distal gut resulted in a genotype-independent elevation of plasma GLP-1 levels after RYGB surgery and might contribute to the weight loss after RYGB. However, the role of GLP-1 in body weight loss after RYGB surgery has been questioned. Both a pharmacological and a genetic loss-of-function approach provided no support for a role of GLP-1 or PYY in the RYGB-induced body weight loss

(Ye et al., 2014). In contrast, a human study showed that combined blockage of GLP-1 and PYY actions increased food intake after RYGB (Svane et al., 2016).

The α-gustducin-independent nature of the increased plasma GLP-1 levels is in contrast with a previous study in *α-gust*$^{-/-}$mice which reported an attenuated RYGB-enhanced GLP-1 secretion (Mokadem et al., 2014). However, Mokkadem *et al*. measured GLP-1 levels after an oral glucose load, while we studied GLP-1 levels after a liquid meal (Nutridrink®) (Mokadem et al., 2014).

In agreement with previous studies, RYGB surgery increased meal-induced PYY levels (Sweeney and Morton, 2014). The effect was more pronounced in *α-gust*$^{-/-}$mice. Nevertheless, only WT mice displayed an increased colonic L-cell number, while PYY mRNA levels were genotype-independently increased. Since the increase in L-cell number is not in line with the observed plasma levels, an altered secretion pattern may be responsible for the elevated plasma PYY levels. For instance, the selective increase in SCFA levels in *α-gust*$^{-/-}$mice may trigger PYY release, explaining the genotype-dependent increase in PYY levels.

Endogenous PYY release is known to suppress electrolyte secretion.(Panaro et al., 2014). The higher PYY levels in *α-gust*$^{-/-}$ mice were indeed accompanied by a less pronounced increase in ion transport in the common limb. These results indicate that α-gustducin-mediated signaling also plays a role in the increase in ion transport of the foregut.

To summarize, our results argue against a major contribution of gustducin-mediated signaling in the metabolic effects of RYGB. Nevertheless, RYGB activated several regulatory systems in which the gustducin mediated signaling pathway plays a role. This study highlights that nutrients cannot only serve as fuel but may regulate a number of physiological processes after RYGB surgery such as tuning of gut hormone release which is the result of multifaceted intestinal adaptations along the gut. Gustducin-mediated sensing mechanisms regulate L-cell enteroplasticity in a region-dependent manner by increasing L-cell number in both the foregut and distal gut and selectively inducing L-cell differentiation in the foregut. Loss of these mechanisms as observed in *α-gust*$^{-/-}$mice is accompanied with an altered expression of nutrient sensors along the gut and may favor SCFA-induced gut hormone release in the distal gut.

Importantly, these gut hormones could contribute to the observed metabolic improvements after RYGB surgery.

Selective targeting of nutrient sensors along the gut may contribute to our further understanding of the role of these chemosensory mechanisms.

Declaration of interest: The authors have nothing to declare.

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Author contributions: Conceptualization, S.S. and I.D.; Methodology, S.S. and I.D.; Formal Analysis, S.S. Investigation, S.S., M.L., B.A., J.L. and L.V.; Writing – Original Draft, S.S. and I.D.; Writing – Review & Editing, S.S., M.L., B.A., J.L., L.V., R.F., K.V. and I.D.; Funding Acquisition S.S. and I.D.; Resources, R.F., K.V. and I.D.; Supervision, S.S. and I.D.

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5.6 Supplementary material

5.6.1 Supplementary material and methods

5.6.1.1 Roux-en-Y gastric bypass

RYGB surgery was performed as previously described (Seyfried et al., 2012). In brief, surgical anaesthesia was induced and maintained throughout the procedure with isoflurane (2–3 % with O_2). Mice were placed on a heating pad to avoid hypothermia and the abdomen was shaved and disinfected before a 4-cm midline laparotomy was performed. The esophagogastric junction was exposed and the esophagus mobilized. The left gastric vessels and the vagal nerve branches were dissected away from the esophagus to avoid respectively consecutive ischemia of the remaining stomach and damage to the vagal nerve. The small vessels on the cardia were cauterized. The stomach was transected at the gastro-esophageal junction. The native stomach was closed with non-resorbable sutures. At 4 cm distal from the pylorus the jejunum was incised for 2 mm on the antimesenterial side and anastomosed in an end-to-side fashion to the esophagus with two continous, non resorbable sutures. The first 5 cm of the jejunum was used to create a Roux limb. Here, an end-to-side jejunojejunostomy between the biliopancreatic (4 cm) and Roux limb was performed to create the common limb. Hereafter the jejunum was double ligated and transected just proximal of the esophagojejunostomy to create the BPL. The abdominal wall and skin were closed. In the sham groups, the small bowel and the gastro-esophageal junction were mobilized and a gastrostomy (5 mm) on the anterior wall of the stomach was performed. The gastrostomy was closed subsequently. To prevent postoperative pain, all mice received carprofen (3mg/kg) and buprenorphine (0.1mg/kg) twice daily the first 2 days after surgery. A western style diet was continued in all groups for 7 weeks till sacrifice. The first three postoperative days, manually crushed chow together with 20ml of Nutridrink® was provided at the bottom of the cage in order to facilitate food intake.

5.6.1.2 Plasma ghrelin Measurement

Plasma samples were extracted on a SEP-Pak C18 cartridge (Waters Corporation, Milford, MA), vacuum-dried and subjected to ghrelin radioimmunoassay (RIA) as previously described (Janssen et al., 2011). For determination of octanoyl ghrelin a rabbit anti-human ghrelin [1-8] antibody was used which does not recognize desoctanoyl ghrelin. Total ghrelin levels were determined using a rabbit anti-human ghrelin [14-28] antibody, which recognizes both octanoyl and desoctanoyl ghrelin.

5.6.1.3 Plasma Glucagon-Like Peptide-1, Peptide YY, insulin and leptin measurement

Samples were analysed for plasma GLP-1 (GLP-1(7-36)amide and GLP-1 (7-37)) using the Active GLP-1 (ver. 2) Kit (Mesoscale Discovery) and plasma leptin and serum insulin levels were determined using the Mouse metabolic Kit (Mesoscale Discovery), according to the manufacturer's instructions. Plasma PYY levels (PYY(1-36) and PYY(3-36)) were determined using a PYY ELISA (Phoenix pharmaceuticals), according to the manufacturer's instructions.

5.6.1.4 Oral Glucose Tolerance Test

An Oral glucose tolerance test (OGTT) was performed 2 weeks before and 5 weeks after surgery. Mice were fasted for 6 hours and gavaged with 2g/kg D-glucose. Tail vein glucose levels were measured at 0, 15, 30, 60 and 120 minutes after glucose administration with a glucometer (Glucomen LX®, A. Menarini Diagnostics) and serum was collected after centrifugation for 7 min at 2000 rpm (4°C) and subsequently snap frozen in liquid nitrogen for insulin measurements. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the equation $[(G_0 \times I_0)/405)]$, where G_0 and I_0 refer to 6-h fasting plasma glucose and insulin values.

5.6.1.5 Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was isolated from mouse tissue segments using the RNeasy kit (Qiagen). Isolated RNA was treated with Turbo DNAfree kit (Ambion) and was reversed transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen). The real-time PCR reaction was performed as described previously, using the Lightcycler 480 (Roche Diagnostics) with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics). Results were expressed relative to the geometric mean of the normalized expression of the three most stable housekeeping genes tested; hypoxanthine phosphoribosyltransferase 1 (Hprt1), ribosomal protein L13a (RPL13a) and β-actin. The following primers were used

Gene	Forward primer	Reverse primer
RPL13a	CACTCTggAggAgAAACggAAgg	gCAggCATgAggCAAACAgTC
β-Actin	gATCTggCACCACACCTTCTAC	TggATggCTACgTACATggCTg
Hprt1	TCAgTCAACgggggACATAAA	ggggCTgTACTgCTTAACCAg
ghrelin	CCAgAggACAgAggACAAgC	ACATCgAAgggAgCATTgAA
GOAT	ATTTgTgAAgggAAggTggAg	CAggAgAgCAgggAAAAAgAg
proglucagon	gAggAgAACCCCAgATCATTCC	gTggCgTTTgTCTTCATTCATC
PYY	CAgTggTgAAgACTCCCCCAAg	TgAACACACACAgCCCTCCAg
SGLT1	CggAAgAAggCATCTgAgAA	AATCAgCACgAggATgAACA
GLUT2	TCTTCACggCTgTCTCTgTg	AATCATCCCggTTAggAACA
TAS1R3	CAggCAgTTgTgACTCTgTTg	TgCgATgCAgATACCTCgTg
LPAR5	gCTgTCgTCTATTCgTCTggC	CgAAgCACAgCAggAAgATg
FFAR2	CCCTgTgCACATCCTCCTgC	gCgTTCCATgCTgATgCCCg
FFAR3	TgTCCAATACTCTgCATCTgT	AggTCCgAAATggTCAggTT
FFAR4	gTCCCATCATCATCACCATCC	gATggCCAgATgACCAggTC
Occludin	gACTgggTCAgggAATATCCACC	AgCAgCAgCCATgTACTCTTCAC

5.6.1.6 Histology and Immunohistochemistry

Gastric or colonic tissues and segments from the RL and BPL, or corresponding segments were sampled as illustrated (Fig. 3a). Samples (5 mice/group) were immediately fixed with paraformaldehyde (4%) for 2h (4°C) followed by cryoprotection in 30% sucrose at 4°C overnight.

Cryostat sections (8 µm) were incubated for 2 h in 0.1 M phosphate-buffered saline (PBS) containing 10% donkey serum and 0.3% Triton X-100 and incubated with one of the following primary antibodies: rabbit anti-octanoyl ghrelin (1/500 for limbs and 1/1000 for gastric tissue, Ab5004, in-house developed), goat anti-ghrelin (1/1000, sc-10368, Santa Cruz), goat anti-GLP-1 (1/150, SC-7782, Santa Cruz Biotechnology) or rabbit anti-PYY (1/1000, Ab22663, Abcam). Substitution of the primary antibody with PBS was used as a negative control. Subsequently, tissues were incubated with secondary antibody for 2 hours, after washing: donkey anti-rabbit Alexa488 (1/1500, A21206, Thermofisher), anti-goat Alexa594 (1/1000, A11058, Thermofisher) or donkey anti-rabbit AMCA (1/250, 711-225-152, Jackson Immuno Labs). For the double-immunofluorescence staining, after being incubated with the secondary antibody and washed three times, sections were incubated for 24 hours at room temperature with the second primary antibody. Subsequently, tissues were incubated with the second secondary antibody for 2 hours. Sections were mounted in Citifluor and visualized under a fluorescence microscope (Olympus BX41). Of each tissue segment three sections

were analyzed using Cell^F Imaging Software (Olympus Soft Imaging Solutions GmbH). Octanoyl ghrelin-, ghrelin-, GLP-1- or PYY-positive cells, were counted in 5 randomly chosen fields (20x) for the stomach and expressed per mm^2, or in the whole section (20x) for the segments from colon, RL, BPL or corresponding segments.

Morphological measurements were performed on hematoxylin and eosin (H&E) sections. Mucosal thickness was measured at 15 randomly chosen representative fields (4x).

5.6.1.7 SCFA Analysis

SCFAs were extracted from the fecal samples using an ether extraction. 50 mg of fecal sample was suspended in 1 ml saturated NaCl (36%). Ether (3 ml), Na_2SO_4 (50 mg), H_2SO_4 (150 µl) and internal standard (50 µl) (2-ethylbutyric acid (Merck)) was added to each sample. The ether layer was collected and the SCFA were analyzed on a gas chromatography–mass spectrometry quadrupole (Finnigan Trace GC, Thermoquest) as previously described.(De Preter et al., 2009) Samples were absolutely quantified using an appropriate calibration curve.

5.6.1.8 Gut Permeability Assays

The distal colon or tissue from the CL or corresponding segment, was dissected in carbogenated Krebs-Ringer buffer (11mM D-glucose). Intestinal segments of approximately 0.5 cm^2 (n=3/mouse) were cut and mounted in modified Ussing chamber (Mussler Scientific Instruments) with an area of 0.017cm^2 for colonic segments, and 0.096cm^2 for segments from CL or the corresponding tissue. Potential difference (PD) was continuously recorded and transepithelial electrical resistance (TEER) ($\Omega x cm^2$) was calculated from the voltage deflections induced by bipolar constant-current pulses of 16 mA every 60 s with duration of 200 ms and was recorded over 2h.

Meanwhile, permeability was measured, by adding fluorescein (376 Da, Sigma Aldrich) to the mucosal side after a calibration period of 30 min. Regular (every 30 min) sampling from the serosal side allowed to detect the degree of mucosal permeability. The fluorescence level ($ng/ml*cm^2$) was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech).

Short circuit current was calculated using the measured TEER and recorded potential difference (voltage) using following formula; Isc = *V/R* where *V* and *R* refer to the measured Voltage (*V*) in micro Ampère and Resistance (*R*) in Ohm.

5.6.1.9 Blinding

Genotype paradigms were blinded during the sample collecting and genotype and treatment paradigms were blinded during the analysis.

5.6.2 Supplementary figures

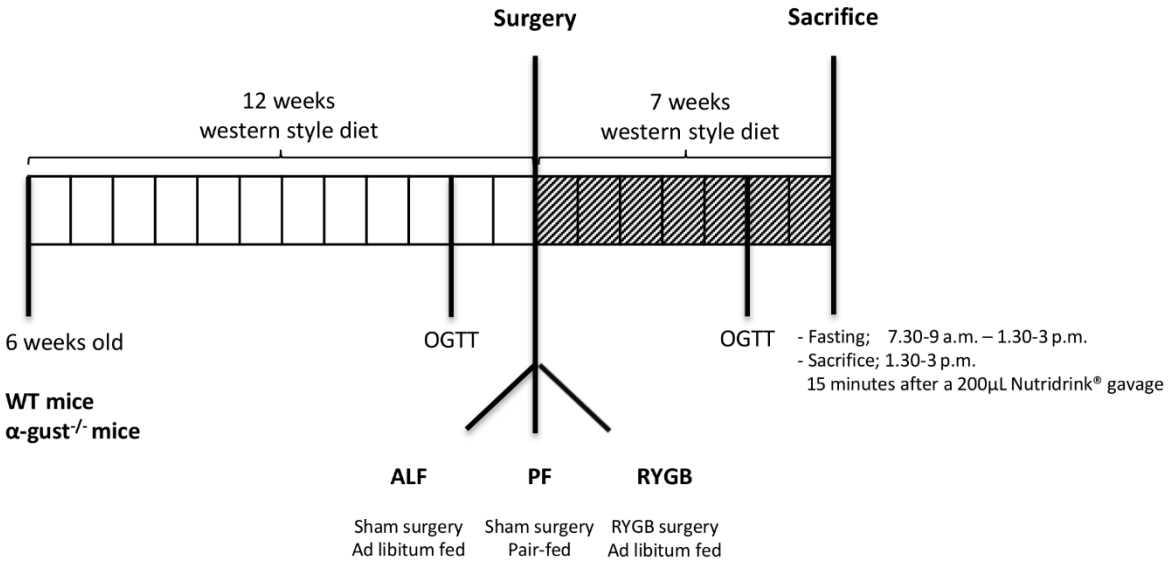

Figure S1. Experimental design. Scheme of the experimental design of the study. WT and α-gust$^{-/-}$mice (6 weeks old) were fed a western diet for 12 weeks. Mice were randomized in three groups: a sham-group fed ad libitum (ALF), a sham-group, pair-fed to the RYGB group (PF), and a RYGB group fed ad libitum fed (RYGB). All mice received the western diet for 7 weeks after surgery till sacrifice. An oral glucose tolerance test was performed 2 weeks before surgery and 2 weeks before sacrifice. Seven weeks post-surgery, (6h) fasted mice (between 7.30-9 a.m. – 1.30-3 p.m. to minimize circadian fluctuations) were gavaged with 200µL Nutridrink®, 15 minutes before sacrifice.

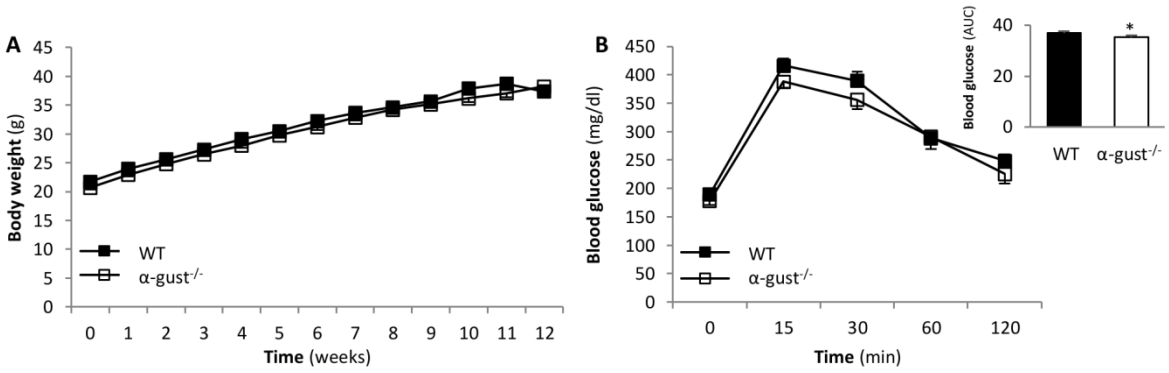

Figure S2. Pre-surgical weight gain and glucose tolerance. (**A**) Pre-surgical time-dependent changes in body weight of WT and α-gust$^{-/-}$mice fed a western style diet (n= 21-23). (**B**) Plasma glucose levels during an OGTT (2g/kg) in WT and α-gust$^{-/-}$mice fed a western style diet, 2 weeks before surgery (n=21-23).* P<0.05 compared to WT mice.

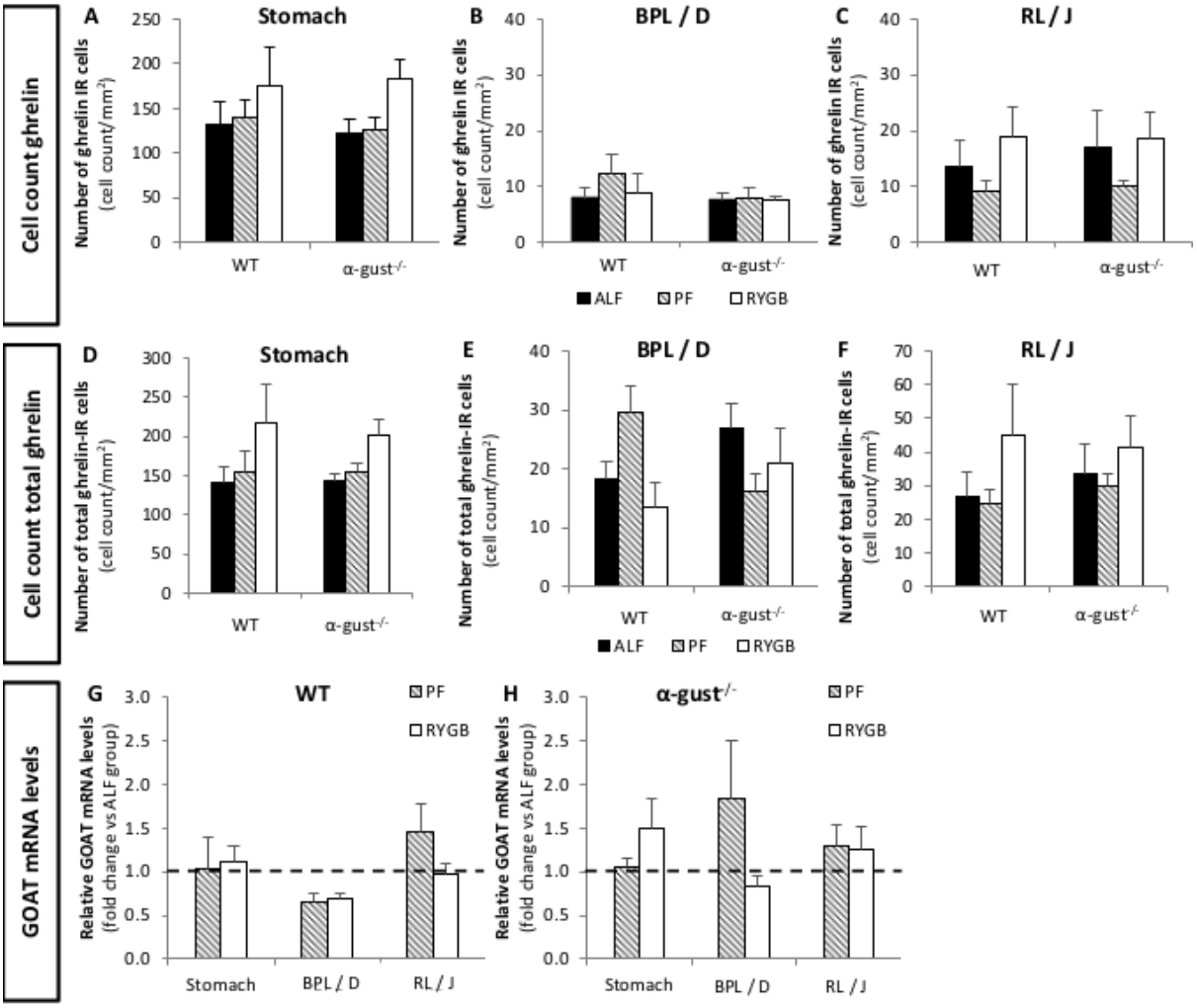

Figure S3. RYGB increased plasma total ghrelin levels in α-gust$^{-/-}$mice. Number of (**A-C**) octanoyl ghrelin and (**D-F**) total ghrelin IR cells in the stomach (**A, D**), BPL (**B, E**),RL (**C, F**) and corresponding small intestine (sham groups) in ALF, PF and RYGB WT and α-gust$^{-/-}$mice (n= 5-8). (**G, H**) Relative mRNA levels of GOAT in the stomach, BPL, RL (RYGB) or corresponding small intestine (sham groups) in PF and RYGB groups in WT and α-gust$^{-/-}$mice (n= 6-8). The dotted line indicates the mean relative GOAT mRNA levels in ALF mice.

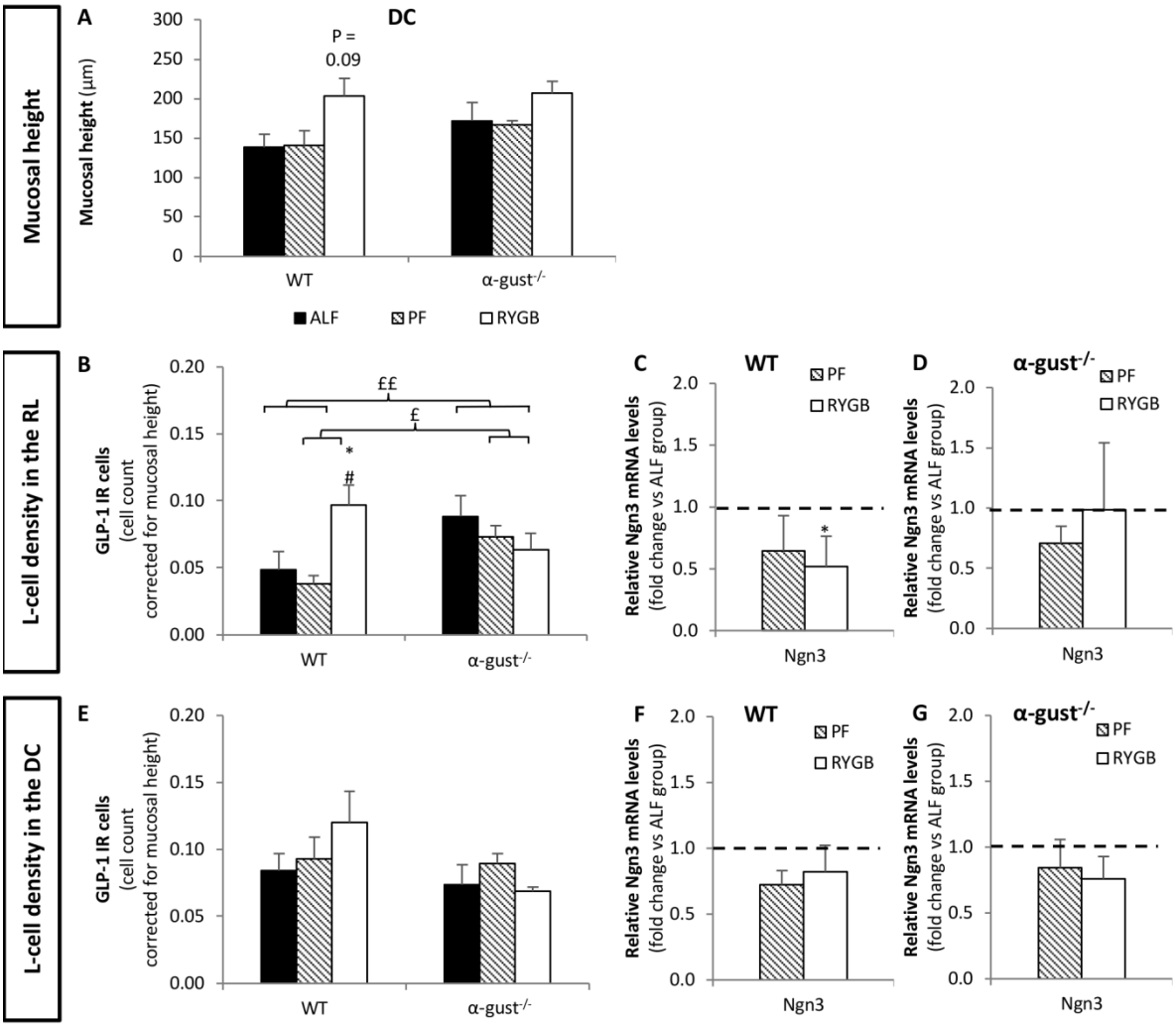

Figure S4. RYGB increased L-cell density in the foregut, but not the distal gut of WT mice in an α-gustducin-independent manner. (**A**) Morphometric analysis showing the mucosal height from sections of the distal colon from ALF, PF and RYGB groups in WT and α-gust$^{-/-}$mice. Density of GLP-1 IR cells in sections of the RL (**B**) and distal colon (**E**) in RYGB groups or corresponding segments (ALF, PF groups) in WT and α-gust$^{-/-}$mice (n= 5). Relative mRNA levels of neurogenin 3 in the RL (**C, D**) and distal colon (**F, G**) or corresponding segment (ALF-PF) in WT and α-gust$^{-/-}$mice (n= 6-8). The dotted line indicates the mean relative neurogenin 3 mRNA levels in ALF mice. * P<0.05 compared to ALF groups. # P<0.05 compared to PF groups. £ P<0.05, ££ P<0.01 genotype*operation effect between the indicated groups

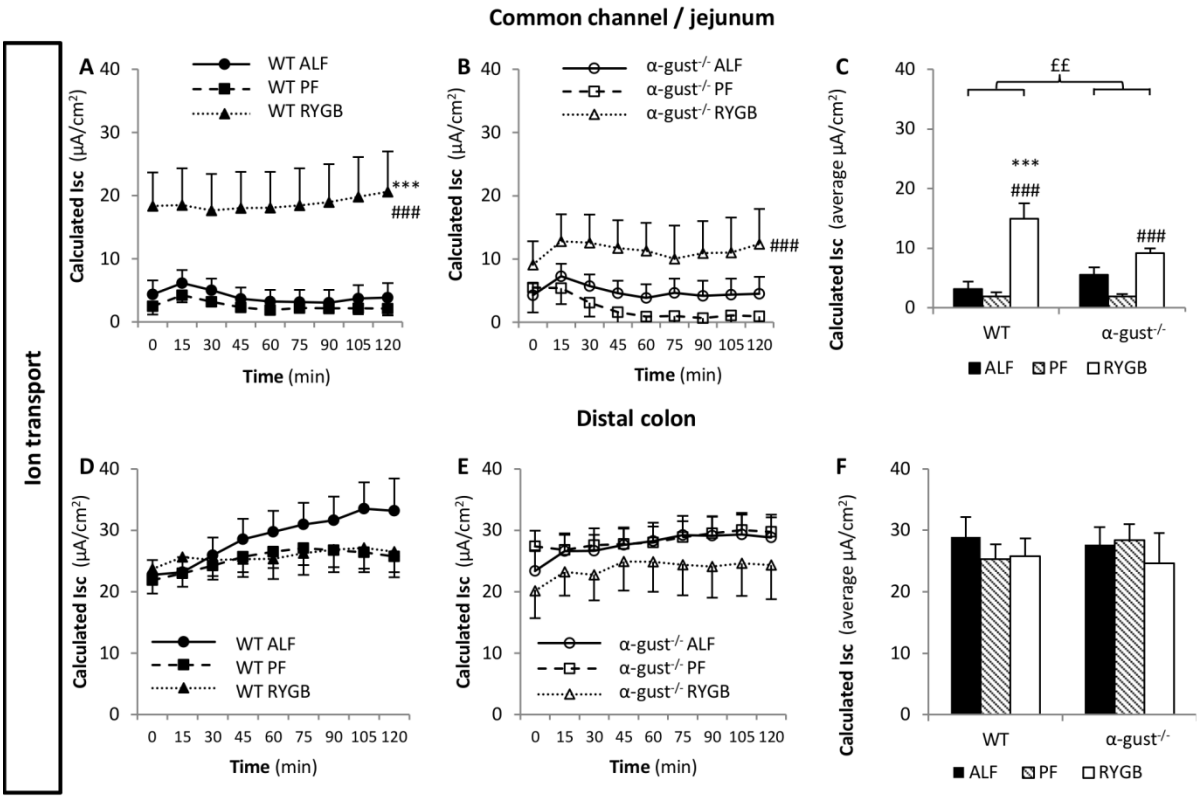

Figure S5. α-gustducin plays a role in the RYGB-induced changes in ion transport across the epithelium (secretory and/or absorption properties) of the diverged small intestine. Equivalent short circuit current (Isc) in the (**A, B**) CL (RYGB groups) and (**D, E**) distal colon or corresponding segment (sham-operated groups) in ALF, PF and RYGB WT and α-gust$^{-/-}$mice (n= 8-9). (**C, F**) Average equivalent short circuit current (Isc). *** P<0.001 compared to ALF groups. ### P<0.001 compared to PF groups. ff P<0.01 genotype*operation effect between the indicated groups.

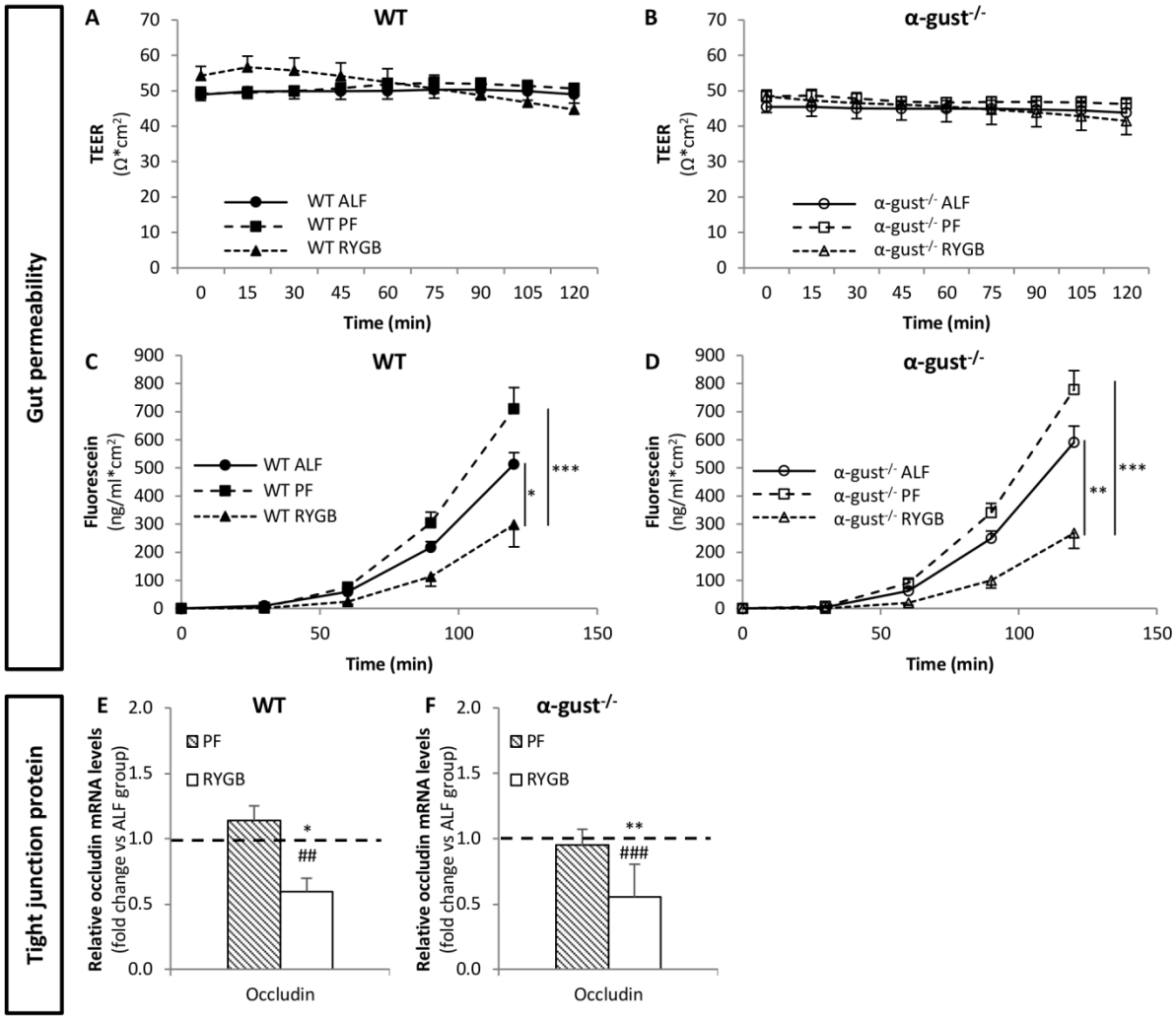

Figure S6. RYGB improved colonic permeability in an α-gustducin-independent manner. (**A, B**) Colonic transepithelial electrical resistance (TEER) 7 weeks after surgery in ALF, PF and RYGB WT and α-gust$^{-/-}$mice (n= 6-8). (**C, D**) Colonic fluorescein passage 7 weeks after surgery in ALF, PF and RYGB WT and α-gust$^{-/-}$mice (n= 6-8). (**E, F**) Relative occludin mRNA levels in the distal colon in PF and RYGB WT and α-gust$^{-/-}$mice (n= 6-9). The dotted line indicates the mean relative occluding mRNA levels in ALF mice * P<0.05, ** P<0.01, *** P<0.001 compared to ALF groups. ## P<0.01, ### P<0.001 compared to PF group.

Chapter 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

6 GENERAL DISCUSSION AND FUTURE PROSPECTS

Several taste receptors (sweet, umami, bitter, fatty acid) and the taste receptor coupled G-protein, α-gustducin, are not only present on taste buds of the tongue but also on EECs and may tune gut hormone release according to the macronutrient composition of the meal. However, the physiological role of these taste receptors on EECs has not yet been fully elucidated. Targeting taste receptors to restore postprandial gut hormone levels (ghrelin, GLP-1, PYY), known to be dysregulated in obesity, may pose a new therapeutic strategy to combat obesity.

This PhD project investigated the functional role of the sweet taste receptor on the ghrelin cell, the potential of targeting sweet/fatty acid taste receptors in the gut by prolonged treatment with artificial/prebiotic sweeteners to prevent body weight gain and the role of α-gustducin-mediated nutrient signaling in the restoration of postprandial gut hormone levels and metabolic improvements after Roux-en-Y gastric bypass (RYGB) surgery.

6.1 Is the omnipresence of α-gustducin limiting its targetability?

Taste signaling is omnipresent throughout the body. For instance, α-gustducin has been reported in several cell types in the gut epithelium including the brush cells, tuft cells and EECs (Sato, 2007, Sternini et al., 2008, Howitt et al., 2016) and in extra-oral sites like the choroid plexus, kidney, keratinocytes, brain, airways, testis, ureter, thyroids, pancreatic duct and adipose tissue (Tomas et al., 2016, Liu et al., 2015, Wolfle et al., 2015, Sternini et al., 2008, Kinnamon, 2012, Gong et al., 2016, Panneck et al., 2014, Hofer and Drenckhahn, 1998, Avau et al., 2015, Ren et al., 2009).

Most of the tissues where the taste transduction machinery is active work as a barrier and it seems that the taste system behaves as a sensor to assess the composition of body fluids. This may then be used to trigger protective responses when hazardous compounds are sensed. For instance, taste receptors in the airways are thought to sense irritating bitter chemicals and to promote protective airway reflexes (Kinnamon, 2012). Furthermore the α-gustducin and TRPM5 dependent signaling pathway in tuft epithelial cells of the gut mediate type 2 cell immune responses during parasite infection to promote worm expulsion (Howitt et al., 2016, Gerbe et al., 2016, von Moltke et al., 2016).

Not only toxic compounds but also nutrients are sensed by extra-oral taste receptors. The sweet taste receptor and short-chain fatty acid receptors FFAR2 and FFAR3 are expressed in mouse and human pancreatic β-cells and our study showed expression of FFAR2 on adipose tissue (Kyriazis et al., 2012, Tang et al., 2015). Kyriazis *et al*. demonstrated that fructose activated sweet taste receptors in isolated mouse islets and synergized with glucose to amplify insulin release. Genetic ablation of TAS1R2 abolished the potentiating effect of fructose on glucose-stimulated insulin release *in vitro* and *in vivo*. Studies in TRPM5$^{-/-}$ mice revealed that this taste receptor signaling pathway increased calcium levels which were dependent on phospholipase C and TRPM5 (Kyriazis et al., 2012). Tang *et*

al. showed that FFAR2 and FFAR3 mediate an inhibition of insulin secretion by coupling to G_i type proteins. Diet-induced obese mice with a genetic ablation of both receptors, on a whole body level or specifically in pancreatic beta cells, had a higher insulin secretion and improved glucose tolerance. However, FFAR2 or FFAR3 deletion in intestinal cells did not alter glucose tolerance (Tang et al., 2015). SCFAs also reduced the intracellular lipolytic activity in differentiated murine 3T3-L1 adipocytes via FFAR2 suggesting a role for FFAR2 on the adipocytes (Ge et al., 2008).

Since α-gustducin and several elements of the chemosensory signaling pathway are so widely expressed throughout the body it is plausible to assume that during our studies signals derived from different sites could cancel each other out. For instance, the stimulatory effect of the gut-derived hormone ghrelin on adipogenesis, could get cancelled out by the increased heat production in α-gustducin$^{-/-}$ mice (Avau et al., 2015). This might explain why the phenotype of WT and full body α-gustducin knockout mice did not present many different features. Nevertheless, lean α-gustducin$^{-/-}$ mice did show lower basal ghrelin levels while diet-induced obese α-gustducin$^{-/-}$ mice tended to have lower GLP-1 and insulin levels.

For the tongue it has been reported that the reduced preference to sweet compounds in two-bottle preference tests in α-gustducin$^{-/-}$ mice is accompanied by reduced responses of the chorda tympani and glossopharyngeal nerves to sweet compounds (Danilova et al., 2006). These nerves together with the vagus innervate the nucleus tractus solitarius. It is therefore tempting to speculate that their reduced signaling in α-gustducin$^{-/-}$ mice also translates in reduced activation of reflex circuits that contribute to the autonomic nerve activation of, for instance, the pancreas and EECs. A role for the autonomous nervous system in the regulation of gut peptide release has been shown for the cephalic insulin response to a meal (Herath et al., 1999) and the preprandial rise in ghrelin levels (Mundinger et al., 2006, Zhao et al., 2010). Thus, the interplay between oral taste perception and activation of the autonomic nervous sytem could contribute to the alterations in gut hormone levels in α-gustducin$^{-/-}$ mice.

6.2 Gut sweet taste receptors: the answer to obesity?

In this PhD study we showed for the first time that the canonical sweet taste receptor signaling pathway does not play a functional role in the effect of sweeteners on ghrelin release. In contrast, an important role for sweet taste receptor signaling in glucose-induced GLP-1 release has been claimed from *in vitro* studies in the human NCI-H716 cell line, duodenal isolated villi and duodenal minced tissues from WT and α-gust$^{-/-}$ mice (Jang et al., 2007). *In vivo*, GLP-1 secretion was blunted in α-gust$^{-/-}$ and TAS1R3$^{-/-}$ mice after an intragastric or intraduodenal glucose administration, respectively (Jang et al., 2007, Kokrashvili et al., 2009a). Blocking sweet taste receptors in the gastro-intestinal tract of healthy volunteers with the sweet taste receptor antagonist lactisole also reduced GLP-1 and PYY secretion after an acute intragastric glucose infusion but to a lesser extent after intraduodenal

administration (Steinert et al., 2011b). Since the TAS1R2 subunit of the sweet taste receptor (TAS1R2-TAS1R3) is not present in the stomach of mice in our study, this is a somewhat counterintuitive result. Other studies using the TAS1R2-lacZ knock-in mouse did also not observe TAS1R2 expression in the stomach (Iwatsuki et al., 2010). In contrast, Koyama *et al*. showed a very low expression of TAS1R2 in the MGN3-1 cell line and primary gastric ghrelin cells using RNA sequencing (Koyama et al., 2016). This suggests that the process of glucose-induced gut hormone secretion is complex and likely to be the result of the summation of different processes, including feedback signals from the small intestine to the stomach. In addition, lactisole did not block the effect of a liquid meal consisting of proteins, fats, and other complex carbohydrates on GLP-1 and PYY release (Gerspach et al., 2011). Thus, although sweet taste receptors may play a role in the effect of glucose on GLP-1 and PYY release, it is likely that this is overruled by other macronutrients in a meal and that blocking sweet taste receptors might not be enough to modulate gut hormone release.

In fact, targeting sweet taste receptors on EECs by acute intragastric administration of artificial/prebiotic sweeteners failed to affect ghrelin release in our study in mice. Similar findings have been reported with an acute intragastric administration of artificial sweeteners in rats (Fujita et al., 2009) and humans (Steinert et al., 2011a, Ma et al., 2009). These findings are at variance with *in vitro studies* in enteroendocrine cell lines where GLP-1 release from NCI-H716 cells was promoted by sucralose (an artificial sweetener) in a dose dependent manner, and blocked by the sweet taste receptor antagonist lactisole or siRNA for α-gustducin (Jang et al., 2007). In the current PhD we also showed effects with sucralose or oligofructose (OFS; a prebiotic sweetener) on ghrelin release *in vitro* in a ghrelinoma cell line and *ex vivo* in segments from both the stomach and jejunum of mice. These findings again underscore that acute activation of sweet taste receptors may not have an important functional role *in vivo* despite promising *in vitro* results.

Long-term administration of sucralose did also not affect ghrelin, GLP-1 and PYY levels in mice on a high-fat diet and did not improve body weight gain nor glucose intolerance. Thus targeting sweet taste receptors on a long-term basis does not seem to be relevant from a therapeutic point of view. It is important to notice though that not all sweet compounds bind to the same binding pocket of the sweet taste receptor heterodimer (DuBois, 2016). Carbohydrates and artificial sweeteners are hence likely to activate a different signaling cascade and although artificial sweeteners may not induce physiological effects, carbohydrates may still signal via the sweet taste receptor. Therefore performing functional binding studies may be important before any conclusions are drawn on the possible effect of new sweeteners on metabolic parameters.

Nevertheless, this does not exclude that sweet taste receptor expression might be altered during disease or that mutations can occur in sweet taste receptors that increase the risk to develop obesity or diabetes. On the tongue, several studies reported an increase in sweet taste threshold perception

in patients with T2DM (Gondivkar et al., 2009, Wasalathanthri et al., 2014) as well as in type 1 diabetes (Khobragade et al., 2012). This taste abnormality may lead to a preference for sweet-tasting foods, thereby exacerbating hyperglycemia. In the gut, we showed that neither long-term administration of sweeteners, nor RYGB surgery affected TAS1R2 mRNA levels in mice. Young *et al*. showed that changes in basal glycemia do not change sweet taste receptor expression in humans. However, the expression of the sweet taste receptor subunit, TAS1R2, was increased in response to luminal glucose during euglycemia in both healthy volunteers and non-obese patients with T2DM. Furthermore, TAS1R2 was downregulated in response to luminal glucose during hyperglycemia in healthy volunteers but increased under the same conditions in non-obese patients with T2DM. This defect enhanced glucose absorption in type 2 diabetic patients and may exacerbate postprandial hyperglycemia (Young et al., 2013).

Previous studies suggested a relationship between the activation of the TAS1R2-TAS1R3 receptor and the expression of SGLT1 (Margolskee et al., 2007). We reported that long-term (8 weeks) administration of equisweet concentrations of the sweetener OFS but not sucralose, followed by the administration of a single glucose bolus, decreased the expression of the glucose transporters in the small intestine. Surprisingly, this downregulation did not affect blood glucose levels. In contrast, 7 weeks after RYGB surgery the expression levels of the glucose transporter were increased in the limb with increased exposure to undigested nutrients and thus also to carbohydrates. This upregulation was accompanied by increased peak glucose levels. Thus, it seems that an increased exposure to sweeteners and carbohydrates differentially regulate the expression levels of glucose transporters.

The glucose transporter SGLT1 may play a role as a glucose-sensor on the L-cell, enabling this transporter to modulate gut hormone release (Reimann et al., 2008). This hypothesis is supported by both a pharmacological (by using the SGLT1 antagonist phloridzin) (Parker et al., 2012) and genetic loss-of function approach ($SGLT1^{-/-}$ mice) (Gorboulev et al., 2011). Recently the correlation between glucose transport, SGLT1 expression and gut hormone release has been investigated in lean and obese volunteers. Nguyen *et al*. showed that obese patients have a higher SGLT1 expression which was correlated with higher glucose absorption rates and plasma insulin and GIP levels compared to lean subjects. In contrast, obesity nor glucose infusion altered TAS1R2 levels (Nguyen et al.). Surprisingly the increase in glucose absorption and SGLT1 expression levels was associated with lower glucose-induced GLP-1 levels. This suggests that the increased proximal glucose absorption will result in a reduced glucose exposure in the distal intestine leading to a diminished glucose-stimulated GLP-1 release.

$SGLT1^{-/-}$ mice displayed decreased blood glucose levels, increased delivery of glucose to the distal small intestine/caecum and increased GLP-1 release, highlighting SGLT1 as a therapeutic target. However, these mice also suffer from glucose and galactose malabsorption, characterized by severe diarrhea (Powell et al., 2013a). To avoid gastro-intestinal side effects, most pharmaceutical

companies focused on the renal glucose transporter SGLT2, of which several selective inhibitors are approved for the treatment of T2DM (Rosenwasser et al., 2013). However, partial blockage of SGLT1, as observed in heterozygous SGLT1$^{-/-}$ mice, increased glucose disposal to the distal small intestine and caecum and increased GLP-1 secretion without inducing the gastro-intestinal side-effects (Powell et al., 2013a). These results opened a therapeutic window for achieving glycemic efficiency with a dual antagonist for SGLT1 and SGLT2. Indeed, treatment with Sotagliflozin/LX4211, a dual SGLT1 and SGLT-2 antagonist for 28 days improved glucose tolerance, increased plasma GLP-1 and PYY levels, and tended to decrease body weight in T2DM patients compared to placebo (Zambrowicz et al., 2012). A longer, 12 week treatment with Sotagliflozin/LX4211, also improved glycemic control and decreased body weight in inadequately controlled type 2 diabetic patients on metformin monotherapy (Rosenstock and Ferrannini, 2015). Powell *et al*. showed that the effect of Sotagliflozin/LX4211 on glucose absorption and GLP-1 and PYY release is due to the blockage of SGLT1 and not SGLT2 in mice (Powell et al., 2013b).
Collectively these findings suggest that intestinal glucose transporters, rather than sweet taste receptors play an important role in the regulation of gut hormone levels in response to glucose. Therefore pharmacological inhibitors for the intestinal SGLT1 transporter, or endoscopic devices that prevent carbohydrate exposure to the proximal intestine and induce a higher carbohydrate exposure to GLP-1 containing L-cells in the distal intestine, may pose potential strategies in the treatment of obesity and T2DM.

We conclude that despite promising results in other models, our results and data in humans currently do not support the concept that acute consumption of low calorie sweeteners can reduce food intake through modulation of gastrointestinal hormones. In contrast, therapeutic modulation of glucose transporters may pose a new strategy for anti-obesity treatments.

6.3 Gut sensing: the emerging role of the distal gut

6.3.1 Short-chain fatty acids in the interplay between diet and energy balance

Long-term administration of the prebiotic sweetener OFS, but not of “equisweet” concentrations of the artificial sweetener, sucralose, decreased body weight gain and the accumulation of fat pad mass in our study. These results highlighted that the effects observed after OFS administration were not mediated through sweet taste receptor activation but likely through the produced SCFAs and thus may involve FFAR2/3. SCFA supplementation may directly decrease fat storage since Lin *et al.* showed that dietary supplementation of butyrate and propionate in mice completely blocked HFD-induced weight gain, while acetate supplementation led to a 40% suppression of excess weight gain (Lin et al., 2012, Lu et al., 2016). SCFAs have also been shown to reduce lipolysis and increase adipogenesis in cultured adipocytes of WT, but not of FFAR2$^{-/-}$ mice, indicating a role of FFAR2 signaling in adipocyte metabolism (Hong et al., 2005, Ge et al., 2008). The reduced body fat after OFS

administration in our study was also accompanied by an increase in FFAR2 expression levels in fat depots, strengthening the hypothesis of a role for FFAR2 signaling on adipose tissue.

FFAR2/3 signaling has also been shown to be mediate gut hormone release. Acetate and propionate inhibited ghrelin secretion through FFAR2 from gastric X/A cells, harvested from ghrelin-hrGFP reporter mice (Engelstoft et al., 2013).

Tolhurst *et al*. showed that propionate and acetate trigger GLP-1 release from primary colonic cultures from WT mice, and that this effect was markedly or slightly diminished in cultures from FFAR2$^{-/-}$ mice and FFAR3$^{-/-}$ mice, respectively (Tolhurst et al., 2012). Furthermore, Brooks *et al*. showed that FFAR2 is important for the body weight loss after supplementation of inulin and that the prebiotic inulin can increase the density of PYY-containing cells and circulating PYY levels through FFAR2 activation (Brooks et al., 2017). In contrast, Lin *et al*. showed that an acute dose of butyrate, but not of acetate or propionate, increased GLP-1 and PYY levels in WT mice which was attenuated in FFAR3$^{-/-}$ mice (Lin et al., 2012). FFAR2$^{-/-}$ and FFAR3$^{-/-}$ mice also showed reduced basal and glucose-induced GLP-1 levels (Tolhurst et al., 2012).

However, the decreased body weight gain and fat pas mass after long-term OFS supplementation in our study was accompanied by a decrease in plasma GLP-1 levels and no effect on plasma PYY or ghrelin levels. The discrepancy between the SCFA-induced GLP-1 release in the acute experiments and the decreased glucose-induced GLP-1 release in our long-term study may indicate a time-related effect of SCFAs on gut hormone release. Indeed, Chambers *et al*. showed that in contrast to an acute colonic administration, long-term colonic administration of inulin-propionate in humans did not alter GLP-1 plasma levels. This indicates that the acute effects are not fully representative for the effects of long-term administration (Chambers et al., 2015).

Furthermore, our results highlight the importance of the route and timing of SCFA administration. In our study, intragastric administration of a specific dose of OFS (300mg/day), one hour before lights off, improved body weight but not glucose tolerance in HFD fed mice, over a course of 8 weeks. In contrast, Cani *et al*. showed that continuous feeding of a HFD supplemented with 10% OFS (±300mg/day) for 4 weeks, improved both body weight and glucose homeostasis (Cani et al., 2006).

Furthermore, feeding *ob/ob* mice a HFD while supplementing the drinking water with 300mg OFS/day for 8 weeks, improved glucose homeostasis but not body weight, although it did reduce fat mass and food intake (Everard et al., 2011). However, feeding diet-induced obese mice a HFD while supplementing the drinking water with 300mg OFS/day for 8 weeks, did improve glucose homeostasis and body weight (Everard et al., 2011).

Targeted delivery could also be a useful strategy to supplement SCFAs since acute colonic infusion of propionate in rodents has been shown to stimulate the secretion of the satiety hormones GLP-1 and PYY through FFAR2 signaling (Psichas et al., 2015). Furthermore, Van der Beek *et al.* investigated the difference between acute proximal and distal colonic infusions of acetate on metabolic parameters in

overweight/obese men. Targeted distal colonic infusions of acetate increased fasting fat oxidation, peptide PYY levels and postprandial glucose and insulin concentrations, while proximal colonic infusions showed no effect on substrate metabolism or circulating hormones, highlighting the role of the delivery site (van der Beek et al., 2016). Inulin-propionate esters were developed and tested as site-specific delivery vehicles for colonic propionate delivery (Polyviou et al., 2016). This inulin-propionate ester reduced weight gain, intra-abdominal adipose tissue distribution, intrahepatocellular lipid content and prevented the deterioration in insulin sensitivity compared to the inulin-control group after 24 weeks (Chambers et al., 2015).

Targeted colonic delivery of acetate/propionate or selective agonists targeting FFAR2/3 in the distal colon or FFAR2 in the adipose tissue might be an alternative to treat obesity.

6.3.2 Is our taste manipulated by the gut microbiota?

Obesity and metabolic disorders are associated with an altered microbial composition and ecology (D'Aversa et al., 2013). Several (Turnbaugh et al., 2009, Ley et al., 2006), but not all (Walters et al., 2014) studies reported a decrease in the fraction of Bacteroidetes species relative to Firmicutes species in obese versus lean individuals. These ratios depend on the caloric balance of the individuals studied, since weight loss increases the relative portion of Bacteroidetes species and microbial diversity (Ley et al., 2006, Cotillard et al., 2013).

Both prebiotic treatment (Delzenne et al., 2011a) and RYGB surgery (Aron-Wisnewsky and Clement, 2014, Kong et al., 2013) have been shown to have beneficial effects on gut microbiota composition. However, in contrast to prebiotic sweeteners, daily administration of artificial sweeteners in healthy volunteers who do not normally consume HIS or HIS-containing foods, induced dysbiosis resulting in a deteriorated glucose intolerance (Suez et al., 2014). Several studies in germ-free mice have shown that the presence and diversity of the different gut microbes plays an active role in the glucose- and energy homeostasis of the host; 1) germ-free mice are leaner than normal mice (Backhed et al., 2004) and are resistant to diet induced obesity from a high-fat or western style diet (Rabot et al., 2010, Backhed et al., 2007), 2) transfer of intestinal microbiota from lean mice to gnotobiotic mice results in weight (fat) gain despite decreased energy intake and increased EE (Backhed et al., 2004), 3) colonization of germ-free mice with the microbiota from obese mice resulted in a greater increase in total body fat compared with germ-free mice colonized with the microbiota from lean mice (Turnbaugh et al., 2006), 4) artificial sweeteners induced dysbiosis causing glucose intolerance (Suez et al., 2014).

This gut microbiota might also be able to modulate the expression of taste receptors on the tongue to alter food preference, and in the gut to alter gut hormone release. Germ-free mice showed an increased lingual CD36 expression, which correlated with an increased preference and caloric intake from fat (Duca et al., 2012). Furthermore, germ-free mice also consumed more of a sucrose solution

compared to control mice, although their preference for sucrose and lingual TAS1R3 and SGLT1 expression was similar (Swartz et al., 2012). The increased consumption of fat and carbohydrates could be an adaptation to the chronic energy-deficient state.

In the gut, germ-free mice showed reduced protein levels of the satiety peptides CCK, GLP-1 and PYY which were accompanied by a decreased intestinal expression of the fatty acid sensors FFAR1, FFAR2, FFAR3, FFAR4 and CD36 and an upregulation of the glucose/umami sensors TAS1R3 and SGLT1 (Swartz et al., 2012, Duca et al., 2012). These findings suggest an interaction between the gut microbiota, gut peptide levels and taste receptors. However, the exact link between these observations and the translation to therapeutically exploitable strategies remains to be investigated.

6.4 The future role of gut hormones in the treatment of obesity

6.4.1 Diet: friend or foe of enteroendocrine cells

The restauration of gut hormone profiles after RYGB surgery is accompanied by structural changes in gut morphology and function due to enteroplasticity.

Intestinal enteroplasticity can beneficially affect the glucose-and energy metabolism. Several mechanisms of action have been proposed for the observed metabolic improvements associated with intestinal enteroplasticity after bariatric surgery; 1) earlier work suggests that the enteroplasticity increases intestinal gluconeogenesis which may decrease food intake and restore glucose homeostasis by compensating for the blunted hepatic gluconeogenesis (Troy et al., 2008), 2) a study in rats suggested that the enteroplasticity results in a decreased glucose absorption in the roux limb after RYGB surgery, resulting in blunted postprandial glucose levels (Stearns et al., 2009) and 3) Saeidi *et al.* reported that after gastric bypass the intestine becomes an important tissue for glucose disposal to meet the anabolic demands of the intestinal tissue growth that occurs after bariatric surgery (Saeidi et al., 2013).

Furthermore, this enteroplasticity seems to be extended to the enteroendocrine cell lineage in both the small intestine and distal part of the intestine. In the small intestine the enteroplasticity was accompanied by an increase in GLP-1 and PYY positive cells after RYGB surgery in our study. In previous studies in rats, RYGB surgery also increased the number of GLP-1, CCK, serotonin and PYY containing cells in the common limb (Mumphrey et al., 2013). Therefore this enteroplasticity might result in elevated nutrient-induced gut hormone secretion. Nutrient exposure seems to be able to influence this enteroplasticity since in our RYGB study, the limb which was overexposed to nutrients showed hypertrophy and an increased number of L-cells, while the limb that does not come into contact with nutrients anymore, did not.

RYGB surgery did not increase L-cell density in the distal part of the colon, although it did increase the amount of L-cells in this tissue, which could be due to an increased colonic mucosal height. OFS supplementation also increased the colonic mucosal height in WT mice without affecting L-cell

density. This enteroplasticity might be due to the altered bacterial fermentation. Our results further indicate that gustducin-mediated signaling may play a role in these effects since the observed colonic hypertrophy after RYGB and OFS supplementation was absent in α-gustducin knockout mice.

If nutrient sensors are involved in this enteroplasticity, specific diets might be able to alter intestinal glucose disposal and programming of EECs in order to regulate gut hormone levels. High-fat feeding stimulated the differentiation towards K-cells in the duodenum of Wistar rats (Gniuli et al., 2010). Furthermore, a previous study in mice highlighted the role of nutrient sensing in L-cell differentiation; ob/ob mice did not show altered L-cell density, while mice on a high-fat diet did in both the jejunum and colon. Thus, the lipid-rich diet is needed to trigger changes in L-cell number (Aranias et al., 2015).

SCFAs have also been shown to increase intestinal differentiation of stem cells into L-cells in the lower part of the gut (Delzenne et al., 2007). Furthermore, using FFAR2$^{-/-}$ mice, Brooks *et al.* showed that the fermentable prebiotic inulin acts via FFAR2 to increase the density of PYY containing cells (Brooks et al., 2017).

These results suggest that we might be able to modulate the gut through nutrient-induced intestinal enteroplasticity in both the small intestine and the distal part of the intestine. However, instead of ingesting a high-fat diet, targeting both short-chain and medium/long chain FFARs in the gut may pose an interesting therapeutic strategy to induce enteroplasticity.

6.4.2 Going to the source: gut hormone multi-agonist drug candidates

Obesity has been associated with altered meal-induced gut hormone release. Obese and overweight patients reported attenuated postprandial plasma GLP-1 (Adam and Westerterp-Plantenga, 2005, Verdich et al., 2001) and PYY levels (le Roux et al., 2006, Zwirska-Korczala et al., 2007), while fasting and postprandial plasma ghrelin levels were reported to be lower in obese individuals and show less pronounced meal-related fluctuations (Carlson et al., 2009). Restauration of these gut hormone profiles are thought to contribute to the superior weight loss and improvement in blood glucose levels observed after RYGB surgery. This suggests that gut hormones could be targets for novel obesity and diabetes therapies.

Our study and previous reports highlighted that the metabolic improvements after RYGB surgery are associated with increased plasma GLP-1 and PYY levels, while the effect on plasma ghrelin levels is controversial (Sweeney and Morton, 2014). RYGB surgery is still an invasive technique but mimicking the sustained and enhanced release of GLP-1 and PYY might be a valuable, non-invasive alternative for bariatric surgery.

GLP-1 is currently the most successful gut hormone to be exploited for therapeutic purposes in humans. Liraglutide, a long-working GLP-1 analogue is currently on the market for the treatment of obesity (Pi-Sunyer et al., 2015). PYY administration might also pose an interesting strategy to

decrease body weight, but it can lead to intolerable side effects like nausea and vomiting (Gantz et al., 2007). Ghrelin receptor antagonists and ghrelin-O acyl transferase inhibitors are another group of molecules which have received some interest for development as anti-obesity targets (Schellekens et al., 2010), but none of these candidates have yet been introduced on the market.

A new treatment strategy may be polypharmaceutical agents. Co-administration of oral GLP-1 and PYY in healthy male volunteers reduced total energy intake and fullness at meal onset, but not 24-h energy intake (Steinert et al., 2010). Furthermore, IV co-infusion of GLP-1 and PYY in lean (Neary et al., 2005) and overweight men (Schmidt et al., 2014) reduced energy intake compared to placebo, although the mono-infusions of GLP-1 or PYY did not, demonstrating a synergistic effect.

Preclinical results in rodents also showed that a triagonist binding the GLP-1 receptor, GIP receptor and glucagon receptor has better metabolic effects compared to the respective dual agonists and lowers body weight without a hypoglycemic risk (Finan et al., 2015). The rationale for this triagonist stems from the proven efficiency of GLP-1 agonists in T2DM and obesity, the ability of glucagon to increase lipolysis and thermogenesis (Davidson et al., 1957, Joel, 1966) and the potency of the incretin hormone GIP to enhance the glycemic benefits of GLP-1 (Finan et al., 2013). The metabolic contribution of each of the three independent agonists within the triagonist was demonstrated in genetic loss-of-function models for each of the three receptors (Finan et al., 2015). However, the magnitude of weight loss in a clinical setting and the speed with which it is achieved needs to be thoroughly established in subsequent work. A strategy combining the synergistic effects of GLP-1 and PYY with an antagonist of the ghrelin receptor may offer a future therapeutic approach to combat obesity.

Chapter 7

SUMMARY

7 SUMMARY

The obesity rates continue to rise worldwide and are associated with adverse health problems, including increased risk of T2DM. Excessive weight gain is often considered to be the result of excessive food intake and/or insufficient physical activity. In addition, the food landscape has shifted dramatically over the past several decades and the increased consumption of soft drinks and other sugar-sweetened beverages is considered as a major contributor to the obesity epidemic. Not surprisingly, non-caloric sweeteners have increased in popularity over the years as a mean to facilitate weight loss by reducing the sugar content of meals without affecting its taste. Next to these non-caloric sweeteners, low-caloric prebiotic sweeteners (oligofructose; OFS) have been proposed as functional food ingredients that could improve lipid metabolism and body weight through beneficial effects ascribed to their fermentation products, the short-chain fatty acids (SCFAs). When dietary changes are insufficient, pharmacotherapy can be added, although the risks and modest nature of weight loss that can be achieved with these anti-obesity drugs highlights the need for new treatment strategies. The gastrointestinal tract is an obvious target for new anti-obesity treatment strategies as it coordinates the release of gut hormones, such as the 'hunger hormone' ghrelin and satiety hormones GLP-1 and PYY, to regulate energy uptake and utilization. Restoring postprandial gut hormone levels (ghrelin, GLP-1, PYY), known to be dysregulated in obesity, may play a role in the metabolic improvements after bariatric surgery such as Roux-en-Y gastric bypass (RYGB) surgery. RYGB surgery is an invasive technique that is associated with elevated GLP-1 and PYY levels. Mimicking the sustained and enhanced release of GLP-1 and PYY in a pharmacological manner might be a valuable, non-invasive alternative for bariatric surgery. The magnitude of postprandial gut hormone release depends on the meal composition. Several taste receptors (sweet, umami, bitter, fatty acid) and the taste receptor coupled G-protein, α-gustducin, are not only present on taste buds of the tongue but also on EECs and may tune gut hormone release according to the macronutrient composition of the meal. The physiological role of taste receptors on EECs has not been fully elucidated yet.

In this thesis, we aimed to unravel whether α-gustducin coupled sweet taste receptors (TAS1R2-TAS1R3) play a role in the sensing of carbohydrates and sweeteners by the ghrelin cell. In addition, we elucidated whether intragastric supplementation of artificial sweeteners (sucralose) or prebiotic sweeteners (OFS) can prevent the deleterious effects of a high-fat diet in mice by altering gut hormone release through gustducin-mediated taste receptor activation. As a last aim, we investigated the effect of nutrient rerouting during RYGB surgery on the α-gustducin-mediated signaling pathways that contribute to the metabolic improvements and physiological adaptations along the gut after RYGB surgery.

In **the third chapter** of this thesis we investigated if α-gustducin mediated sweet taste receptor signaling is involved in the sensing of sweeteners by the ghrelin cell in three different experimental models (a ghrelinoma cell line, *ex vivo* intestinal segments, *in vivo* experiments).
The carbohydrate D-glucose and prebiotic sweetener OFS decreased ghrelin release from a gastric ghrelinoma cell line at concentrations physiological to the postprandial luminal fluid. In contrast, the artificial sweetener sucralose increased ghrelin release *in vitro* at a supraphysiological (200mM) concentration. Furthermore, by using pharmacological inhibitors we showed that neither sweet taste receptor activation, nor glucose transport (SGLT1, GLUT family) played a role in the effect of D-glucose, OFS or sucralose on ghrelin release from the ghrelinoma cell line. Ghrelin release from gastric (only containing the TAS1R3 subunit) and jejunal (containing the TAS1R2 and TAS1R3 subunit) segments from WT and α-gust$^{-/-}$ mice, mimicked the *in vitro* effects of the sweeteners in the ghrelinoma cell line to a similar extent in both genotypes. These findings indicate that the effect of D-glucose, OFS and sucralose on ghrelin release is neither α-gustducin nor region-dependent and thus does not involve the α-gustducin coupled TAS1R2-TAS1R3 heterodimer. Intragastric, but not intravenous administration of D-glucose decreased plasma octanoyl ghrelin levels in WT and α-gust$^{-/-}$ mice, indicating that the sensing of D-glucose is polarized and occurs via the luminal side of the X/A cell. In contrast, neither OFS nor sucralose at "equisweet" concentrations affected octanoyl ghrelin release after an intragastric administration in WT or α-gust$^{-/-}$ mice.
In conclusion, our findings indicate that α-gustducin-mediated sweet taste receptor signaling does not play a functional role in the effect of sweeteners on ghrelin release. In contrast to the *in vitro* findings, only acute intragastric administration of D-glucose but not OFS or sucralose affected ghrelin release.

In **the fourth chapter** of this thesis we studied whether daily intragastric administration of equisweet concentrations of an artificial sweetener (sucralose) or a prebiotic sweetener (OFS) for 8 weeks can prevent high-fat diet induced body weight gain, glucose intolerance and impairment of gut permeability, via activation of taste receptors coupled to α-gustducin, using WT and α-gust$^{-/-}$ mice.
Sucralose administration did not modulate gut hormone release nor did it prevent body weight gain or glucose intolerance. Instead we provided evidence that OFS (300mg/day) administration decreased HFD-induced body weight gain with about 20% without improving glucose homeostasis. This effect was not accompanied by a reduced food intake. Furthermore, OFS induced a similar but delayed decrease in body weight gain in α-gust$^{-/-}$ mice, indicating that the α-gustducin mediated signaling pathway did not play a major role in this effect. OFS administration did not affect plasma levels of 'the hunger hormone' ghrelin and 'satiety hormone' PYY, but decreased plasma levels of 'the satiety hormone' GLP-1 in WT mice. These changes in gut hormone levels cannot explain the beneficial effects on body weight. Neither OFS, nor sucralose administration altered the mRNA expression levels of the TAS1R2 or TAS1R3 subunit of the sweet taste receptor in the gastro-

intestinal tract. However, OFS supplementation decreased cecal acetate and butyrate levels, downregulated colonic short chain fatty acid receptor (FFAR2/3) mRNA levels and upregulated FFAR2 in peripheral adipose tissue. These findings suggest that, not sweet taste receptor activation, but enhanced uptake of SCFAs produced by the fermentation of OFS interacting with FFAR2 in peripheral adipose tissue may reduce adipogenesis and lead to the decrease (60%) in fat mass. Moreover, OFS improved the increased colonic permeability which results in metabolic complications in obesity, independent from taste receptors coupled to α-gustducin.

In conclusion, this study provided evidence that despite the controversy in the field, artificial sweeteners are metabolically inert. Furthermore, neither OFS nor sucralose affected TAS1R2 or TAS1R3 mRNA levels, while OFS supplementation altered FFAR2/3 expression levels in the gastrointestinal tract and on adipose tissue. Therefore, not sucralose but OFS and especially the produced SCFAs, are interesting metabolites that could beneficially affect body weight gain.

In **the fifth chapter** we studied the role of gustducin-mediated signaling in the metabolic improvements and intestinal adaptations along the gut after RYGB surgery in obese WT and α-gust$^{-/-}$ mice.

We showed that RYGB surgery decreased body weight in WT and α-gust$^{-/-}$ mice. Furthermore, pair-feeding to the RYGB group induced similar blood glucose and plasma insulin profiles during an oral glucose tolerance test compared to RYGB surgery, indicating that the reduced food intake after RYGB surgery was responsible for the improved glucose homeostasis. Moreover, α-gust$^{-/-}$ mice were partially protected from the diabetogenic properties of a western style diet, highlighting the importance of the gustatory signaling pathway in glucose homeostasis. RYGB surgery increased plasma GLP-1 levels in both genotypes and plasma PYY levels only in α-gust$^{-/-}$mice. Plasma octanoyl ghrelin levels were not affected after surgery. The mechanism behind the postsurgical changes in gut hormone levels seemed to differ between WT and α-gust$^{-/-}$ mice.

In WT mice, nutrients act via α-gustducin to increase L-cell differentiation (in the Roux limb which comes in contact with more undigested nutrients) and L-cell number (Roux limb and colon) after RYGB surgery, in a region-dependent manner. However, this nutrient rerouting did not alter the mRNA expression levels of nutrient sensors in the Roux Limb, nor did it alter bacterial fermentation in the caecum of WT mice. In contrast, α-gust$^{-/-}$ mice did not display an altered L-cell number or L-cell differentiation in the Roux limb or colon. However, α-gust$^{-/-}$ mice did show increased mRNA expression levels of the glucose transporters (SGLT1 and GLUT2) and the protein sensor (LPAR5) in the Roux limb. Furthermore, RYGB surgery changed bacterial fermentation in the caecum of α-gust$^{-/-}$ mice, which showed increased butyrate and propionate levels compared to WT mice. This resulted in decreased colonic FFAR2/3 mRNA levels in α-gust$^{-/-}$ mice. These results suggest that a changed L-cell number and differentiation will be responsible for the increased plasma GLP-1 levels in WT mice. In contrast, alterations in nutrient signaling in the foregut, and altered bacterial fermentation and

short-chain fatty acid sensing in the distal gut of α-gust$^{-/-}$ mice could explain the increased plasma GLP-1 and PYY levels in this genotype. Finally, signaling via α-gustducin plays a role in the increased ion transport of the foregut but not in the improvement in colonic barrier function.

To summarize, our findings do not indicate a major contribution of α-gustducin-mediated signaling in the metabolic effects of RYGB. Nevertheless, RYGB activated several regulatory systems in which the gustducin mediated signaling pathway plays a role. This study highlights that nutrients cannot only serve as fuel but may regulate a number of physiological processes after RYGB surgery such as tuning of gut hormone release which is the result of multifaceted intestinal adaptations along the gut. Importantly, these gut hormones could contribute to the observed metabolic improvements after RYGB surgery.

In conclusion our studies suggest that the canonical sweet taste receptor signaling pathway does not seem to play a major role in the sensing of carbohydrates or sweeteners by the ghrelin cell. Furthermore, *in vivo* targeting of sweet taste receptors on EECs by supplementing artificial sweeteners directly into the stomach for several weeks does not help to prevent the development of obesity nor T2DM. In contrast, prebiotic sweeteners seem to be more promising since their fermentation products target SCFA receptors. On a long term basis their effect on short-chain fatty acid induced gut hormone release does not seem to play a major role in the development of obesity but their capacity to alter expression levels of SCFA receptors on adipose tissue might be relevant. Finally, gustducin-mediated nutrient sensing does not play a role in the effect of RYGB surgery on the energy-and glucose homeostasis, but altered gut hormone levels might.

Chapter 8

SAMENVATTING

8 SAMENVATTING

De prevalentie van obesitas blijft toenemen en is geassocieerd met comorbiditeiten zoals T2DM. Gewichtstoename is vaak te wijten aan een te hoge calorische inname en/of te lage fysieke activiteit. Hiernaast is ons voedselconsumptiepatroon over de laatste decennia sterk veranderd waarbij de toegenomen consumptie van frisdranken en andere met suiker gezoete dranken een belangrijke bijdrage levert aan de obesitas epidemie. Bijgevolg worden niet calorische zoetstoffen meer en meer gebruikt als een middel om gewichtsverlies te bekomen door de hoeveelheid suiker in de maaltijden te vervangen zonder aan smaak in te boeten. Naast deze niet calorische zoetstoffen worden laag calorische prebiotische zoetstoffen (oligofructose; OFS) voorgesteld als functionele voedingsstoffen die het vetmetabolisme en de energiehuishouding kunnen beïnvloeden via hun fermentatieproducten, de korteketenvetzuren. Wanneer veranderingen in voedingsgewoonten niet voldoende zijn in de strijd tegen obesitas, kan een farmacologische behandeling toegevoegd worden. De nevenwerkingen en beperkte efficiëntie van deze geneesmiddelen benadrukken echter de nood aan nieuwe therapeutische strategieën.

Het gastro-intestinaal stelsel is een voor de hand liggend doelwit voor nieuwe anti-obesitas strategieën aangezien het de vrijstelling coördineert van maag-darmhormonen, zoals het 'hongerhormoon' ghreline en de verzadigingshormonen GLP-1 en PYY, die onze energieopname- en verbruik regelen. Het herstellen van postprandiale plasmawaarden van maag-darmhormonen (ghreline, GLP-1 en PYY), die ontregeld zijn in de obese populatie, kan een belangrijke rol spelen in de metabole veranderingen na bariatrische chirurgie zoals Roux-en-Y maagbypass (RYGB). RYGB is een invasieve chirurgische techniek die gepaard gaat met een sterk verhoogde vrijstelling van GLP-1 en PYY. Het farmacologisch nabootsen van deze gewijzigde plasmawaarden van maag-darmhormonen kan een waardevol niet invasief alternatief zijn voor RYGB. De grootte van de postprandiale vrijstelling van maag-darmhormonen hangt af van de samenstelling van de maaltijd. Verschillende smaakreceptoren (zoet, umami, bitter en vetzuren) en het smaak G-proteïne, α-gustducine, zijn niet enkel aanwezig op de smaakpapillen op de tong, maar ook op enteroendocrine cellen (EEC) in het maag-darmstelsel en kunnen daar mogelijks de vrijstelling van maag-darmhormonen afstellen op de samenstelling van de maaltijd. De fysiologische rol van deze smaakreceptoren op EEC is echter nog niet volledig achterhaald.

In dit doctoraatsproject werd nagegaan of zoetsmaakreceptoren (TAS1R2-TAS1R3) gekoppeld aan α-gustducine een rol spelen in het waarnemen van koolhydraten en zoetstoffen door de ghrelinecel. Vervolgens werd nagegaan of intragastrische supplementatie van artificiële (sucralose) of prebiotische (oligofructose) zoetstoffen de nadelige effecten van een hoogvetdieet kunnen voorkomen door maag-darmhormonen vrij te stellen na activatie van gustducine-gekoppelde smaakreceptoren. Ten slotte onderzochten we of het omleiden van nutriënten na RYGB een effect

heeft op gustducine-gemedieerde signaalwegen en of deze bijdragen tot de metabole veranderingen en fysiologische aanpassingen van de darm na RYGB.

In **het derde hoofdstuk** van dit doctoraatsproject werd, in drie verschillende experimentele modellen (een ghrelinoma cellijn, intestinale segmenten van muizen en *in vivo* experimenten) nagegaan, of gustducine-gekoppelde zoetsmaakreceptoren een rol spelen in het waarnemen van zoetstoffen door de ghrelinecel.
De suiker D-glucose en het prebiotische koolhydraat OFS doen de ghrelinevrijstelling in een gastrische ghrelinoma cellijn dalen bij concentraties die fysiologisch zijn aan de postprandiale luminale inhoud, terwijl een suprafysiologische concentratie (200mM) van de artificiële zoetstof sucralose de ghrelinevrijstelling in dit model doet stijgen. Door farmacologische inhibitoren te gebruiken toonden we aan dat noch activatie van de zoetsmaakreceptor, noch glucose transport (via SGLT1, de GLUT familie) een rol spelen in het effect van D-glucose, OFS of sucralose op de ghrelinevrijstelling in de ghrelinoma cellijn. Het effect van glucose en zoetstoffen op de ghrelinevrijstelling van segmenten van de maag (bevat enkel de TAS1R3 subeenheid) en jejunum (bevat zowel de TAS1R2 als de TAS1R3 subeenheid) van zowel WT als α-gust$^{-/-}$muizen was gelijkaardig aan deze bekomen in de ghrelinoma cellijn. Deze bevinding duidt erop dat het effect van D-glucose, OFS en sucralose op ghrelinevrijstelling niet afhankelijk is van α-gustducine, noch regiospecifiek is en bijgevolg niet gemedieerd is via het aan α-gustducine-gekoppelde TAS1R2-TAS1R3 heterodimeer. Intragastrische, maar niet intraveneuze, toediening van D-glucose veroorzaakte een daling van de plasma ghrelinewaarden van WT en α-gust$^{-/-}$ muizen. Hieruit kunnen we concluderen dat het waarnemen van D-glucose door de ghrelinecel gepolariseerd is en aan de luminale zijde van de X/A cel gebeurt. Aan concentraties met een gelijk zoetgehalte veroorzaakte noch OFS noch sucralose na intragastrische toediening een wijziging in de ghrelinewaarden van WT of α-gust$^{-/-}$ muizen. Deze studie toont aan dat activatie van gustducine-gekoppelde zoetreceptoren geen functionele rol speelt in het effect van zoetstoffen op ghrelinevrijstelling. In tegenstelling tot de *in vitro* resultaten kan enkel D-glucose, maar niet OFS of sucralose, de ghrelinevrijstelling *in vivo* beïnvloeden.

In **het vierde hoofdstuk** van deze thesis bestudeerden we of dagelijkse intragastrische toediening van equizoete concentraties van een artificiële (sucralose) of prebiotische (oligofructose) zoetstof gedurende 8 weken het nadelige effect van een hoogvetdieet op lichaamsgewicht, T2DM en darmpermeabiliteit kan voorkomen via activatie van smaakreceptoren gekoppeld aan α-gustducine. Hiervoor werden WT en α-gust$^{-/-}$ muizen gebruikt. De artificiële zoetstof sucralose had geen effect op de vrijstelling van de maag-darmhormonen, noch voorkwam het de toename in lichaamsgewicht of de ontwikkeling van T2DM geïnduceerd door het hoogvetdieet. Desalniettemin deed een éénmaal dagelijkse toediening van OFS (300mg/dag) gedurende 8 weken de toename van lichaamsgewicht, geïnduceerd door een hoogvetdieet, met 20% afnemen, zonder de glucose homeostase te verbeteren. Dit effect ging niet gepaard met een gedaalde voedselinname. Verder induceerde OFS

een gelijkaardig maar vertraagd effect op de toename in lichaamsgewicht in α-gust$^{-/-}$ muizen. Dit wijst erop dat de gustducine-gemedieerde signaalweg geen belangrijke rol speelt in dit effect. OFS toediening beïnvloedde de plasmawaarden van het 'hongerhormoon' ghreline en het 'verzadigingshormoon' PYY niet, maar deed de plasmawaarden van het 'verzadigingshormoon' GLP-1 dalen in WT muizen. Een daling in verzadigingshormoon kan echter het lager lichaamsgewicht na OFS supplementatie niet verklaren.

Daarboven hadden noch OFS noch sucralose een effect op de mRNA expressie van de TAS1R2 of TAS1R3 subeenheid van de zoetsmaakreceptor doorheen het maag-darmstelsel. OFS supplementatie veroorzaakte een daling in het acetaat en butyraat gehalte in het caecum en in de mRNA expressie van de korteketenvetzuurreceptoren, FFAR2/3, in het colon. Daarentegen werd de mRNA expressie van de FFAR2 in het vetweefsel verhoogd. Deze resultaten suggereren dat niet zoetreceptoractivatie, maar wel de toegenomen opname van korteketenvetzuren en hun interactie met FFAR2/3 in perifeer vetweefsel adipogenese kan reduceren om alzo een daling (60%) in vetmassa te induceren. Daarboven verbeterde OFS de gestegen permeabiliteit in het colon, onafhankelijk van smaakreceptoren gekoppeld aan α-gustducine.

Deze studie toont aan dat, ondanks de tegenstrijdige resultaten in het onderzoeksveld, artificiële zoetstoffen toch metabool inert zijn. Daarnaast beïnvloedde noch OFS noch sucralose de mRNA expressieniveaus van TAS1R2 of TAS1R3. Daarentegen beïnvloedde OFS supplementatie wel de FFAR2/3 mRNA expressieniveaus in het maag-darmstelsel en in het vetweefsel. Hieruit kunnen we concluderen dat niet sucralose, maar OFS en voornamelijk de geproduceerde korteketenvetzuren interessante metabolieten zijn die mogelijks een voordelig effect op lichaamsgewicht kunnen hebben.

In **het vijfde hoofdstuk** van dit doctoraatsproject onderzochten we de rol van de gustducine-gemedieerde signaalweg in de metabole veranderingen en intestinale wijzigingen na RYGB in obese WT en α-gust$^{-/-}$ muizen. RYGB deed het lichaamsgewicht van WT en α-gust$^{-/-}$ muizen dalen. Daarnaast induceerde *pair-feeding* dezelfde verbetering in bloed glucose en plasma insuline profielen als RYGB, na een orale glucosetolerantietest. Dit duidt erop dat de gedaalde voedselinname na RYGB verantwoordelijk is voor de verbeterde glucose homeostase. Daarboven waren α-gust$^{-/-}$ muizen gedeeltelijk beschermd tegen het diabetogeen effect van een hoogvet-hoogsuikerdieet, wat het belang van de gustatorische signaaltransductieweg in de glucosehomeostase onderlijnt. RYGB verhoogde de plasmawaarden van GLP-1 in beide genotypes en van PYY enkel in de α-gust$^{-/-}$ muizen. De plasmawaarden van ghreline waren niet beïnvloed. Het mechanisme achter de postoperatieve veranderingen in de plasma waarden van deze maag-darmhormonen leek echter te verschillen tussen de WT en α-gust$^{-/-}$ muizen.

In WT muizen verhoogden nutriënten via gustducine-gemedieerde signaalwegen de L-cel differentiatie (in de *Roux limb*, het deel van de darm dat in contact komt met meer onverteerd

voedsel) en het aantal L-cellen (in de *Roux limb* en distale deel van de darm) na RYGB in een regio-afhankelijke manier. Deze veranderingen waren niet gecorreleerd met veranderingen in mRNA niveaus van de nutriënt sensoren in de *Roux limb,* noch met wijzigingen in de bacteriële fermentatie in het caecum van WT muizen. In tegenstelling tot de WT muizen, vertoonden α-gust$^{-/-}$ muizen geen veranderingen in het aantal of de differentiatie van de L-cellen in de *Roux* limb van het colon. Desalniettemin verhoogde RYGB de mRNA niveaus van de glucosetranssporters (SGLT1 en GLUT2) en de proteïnesensor (LPAR5) in de proximale darm van α-gust$^{-/-}$ muizen. Verder veranderde RYGB de bacteriële fermentatie in het caecum van α-gust$^{-/-}$ muizen, die verhoogde butyraat en propionaat niveaus vertoonden in vergelijking met WT muizen. Dit resulteerde in gedaalde FFAR2/3 mRNA niveaus in het colon van α-gust$^{-/-}$ muizen. Deze resultaten suggereren dat de verandering in het aantal en de differentiatie van L-cellen verantwoordelijk kunnen zijn voor de gestegen plasmawaarden van GLP-1 in WT muizen. In α-gust$^{-/-}$ muizen zal eerder de gewijzigde waarneming in de signaalweg van nutriënten in de proximale darm en de veranderde bacteriële fermentatie en korteketenvetzuur signaalweg in de distale darm de gestegen plasma GLP-1 en PYY waarden in dit genotype verklaren. Tenslotte speelt de gustducine-gemedieerde signaalweg een rol spelen in het toegenomen ionentransport in de proximale darm, maar niet in de verbeterde darmpermeabiliteit in het colon na RYGB.

Samenvattend suggereren onze resultaten dat de gustducine-gemedieerde signaalweg geen belangrijke rol speelt in de metabole veranderingen na RYGB. Desalniettemin activeert RYGB wel verschillende regulatorische systemen waarin de gustatorische signaalweg een rol speelt. Verder toont deze studie aan dat nutriënten niet enkel kunnen dienen als brandstof, maar na RYGB ook een aantal fysiologische processen kunnen regelen zoals het afstemmen van de secretie van maag-darmhormonen, welke het resultaat is van multifactoriële intestinale veranderingen in de darm. Het is belangrijk op te merken dat deze maag-darmhormonen kunnen bijdragen tot de metabole veranderingen na een RYGB operatie.

In conclusie suggereren onze studies dat de zoetsmaakreceptor signaalweg geen belangrijke rol speelt in het waarnemen van koolhydraten of zoetstoffen door de ghreline cel. Verder gaat het *in vivo* beïnvloeden van zoetsmaakreceptoren op EEC, door gastrische toediening van zoetstoffen gedurende verschillende weken, niet bijdragen tot de preventie van obesitas en T2DM. Prebiotische zoetstoffen lijken echter veelbelovend te zijn aangezien hun fermentatieprodukten de korteketenvetzuurreceptoren kunnen activeren. Op lange termijn lijkt het effect van deze korteketenvetzuren op de vrijstelling van maag-darmhormonen geen belangrijke rol te spelen in de ontwikkeling van obesitas, maar hun capaciteit om vetzuurreceptoren op het vetweefsel te activeren kan relevant zijn. Tenslotte speelt de gustducine-gemedieerde signaalweg geen rol in het effect van RYGB op de energie-en glucose homeostase, alhoewel maag-darmhormonen hier mogelijks wel een rol in kunnen spelen.

Chapter 9

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9 REFERENCES

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CURRICULUM VITAE

Sandra Steensels was born on December 29th 1989 in Geel, Belgium. After finishing her high school studies Science-Mathematics at Sint-Jan Berchmanscollege in Mol in 2007, she went on to study Pharmaceutical Sciences at the Catholic University of Leuven. She obtained her bachelor's degree in 2010 and graduated *cum laude* as Master in Pharmaceutical Sciences (Drug Development) in 2012. In September 2012, she started her PhD under supervision of Prof. Dr. Inge Depoortere at the Translational Research Center for Gastrointestinal Disorders (TARGID), Catholic University of Leuven. In the beginning of 2013 she obtained an IWT doctoral fellowship. Her scientific work has contributed to several abstracts and peer-reviewed publications.

LIST OF PUBLICATIONS

Articles in internationally reviewed scientific journals

Steensels S, Lannoo M, Swennen T, Thijs T, Avau B, Laermans J, Vancleef L, Farré R, Verbeke K, Depoortere I. *The gustatory signaling pathway mediates the metabolic reprogramming after Roux-en-Y gastric bypass surgery in mice*. J Endocrinol. 2017 Mar 1;232(3):363-376

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